GENETICS OF TYPE VI SECRETION AND NATURAL TRANSFORMATION IN *VIBRIO CHOLERAE*

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GENETICS OF TYPE VI SECRETION AND NATURAL TRANSFORMATION IN *VIBRIO CHOLERAE*

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To the unknown scientist,

Plugging away in the dark so others may see the light.

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LIST OF SYMBOLS AND ABBREVIATIONS

AI-2	autoinducer-2
cAMP	cyclic adenosine monophosphate
ASW	artificial sea water
Aux	Auxiliary
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAI-1	cholera autoinducer-1
CBP	chitin binding protein
CCR	carbon catabolite repression
cfu	colony-forming unit
Cm	chloramphenicol
CRE	competence regulatory element
CRP	catabolic repressor protein
CDS	coding sequence
CT	cholera toxin
CytR	cytidine repressor protein
DNA	deoxyribonucleic acid
DUS	DNA uptake sequence
GFP	Green fluorescent protein
GlcNAc	β-1,4-linked *N*-acetylglucosamine
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
h	hours
HCD	high cell density
Hyb.	hybrid
HGT	horizontal gene transfer
IM	inner membrane
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
LCD	low cell density
LB	Luria-Bertani medium
M	molar
µg	micrograms
mg	milligrams
mM	millimolar
µL	microliter
mL	milliliter
µm	micrometer
N.S.	not significant
OD	optical density
OM	outer membrane
PCR	polymerase chain reaction
Qrr	quorum regulatory RNA
QS	Quorum sensing
RFP	Red fluorescent protein

RLU	relative light units
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
sRNA	small ribonucleic acid
RNAP	RNA polymerase
RNA-seq	RNA sequencing
Spec	spectinomycin
Str	streptomycin
TF	transformation frequency
T6SS	type VI secretion system
T6S	type VI secretion
UTR	untranslated region
WT	wild type
Xgal	(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

SUMMARY

The facultative waterborne pathogen *Vibrio cholerae* transitions between its human host and the environment where it colonizes chitinous surfaces in aquatic settings. Growth on chitin coordinates the induction of sets of genes for 1) chitin utilization; 2) a type VI secretion system that allows contact-dependent killing of neighboring bacteria; and 3) DNA uptake by natural transformation, which is a mechanism for horizontal gene transfer. This thesis describes the regulatory network controlling these behaviors in *V. cholerae* and the consequences of their coordinate regulation. Results from high-throughput RNA sequencing (RNA-seq) show that transcription factor CytR is one of four positive regulators comprising the chitin-induced regulatory network. A combination of genetic and phenotypic assays reveal the four regulators TfoX, HapR, QstR and CytR control each behavior in a distinct manner in a commonly used clinical reference strain of *V. cholerae*. Whole genome sequencing and bioinformatics analyses of a set of strains isolated from diverse sources reveal novel type VI secretion system components present in environmental, but not clinical isolates. Finally, I show that chitin-induced natural transformation can facilitate horizontal gene transfer of distinct type VI secretion system genes between strains. Horizontally acquired effector-immunity proteins are functional in the new genetic background and can be employed in antibacterial antagonism against parental cells and simultaneously protect against attacks by the donor cells. This thesis sheds light on diverse behavioral adaptations that allow this important human pathogen to spread and persist in the environment.

CHAPTER 1. Introduction

1.1 *Vibrio cholerae* and Chitin

The human pathogen *Vibrio cholerae* is the causative agent of diarrheal disease cholera. This water-borne pathogen is primarily a natural inhabitant of aquatic environments within marine and brackish water systems and commonly forms biofilms on multiple surfaces, such as planktonic organisms, chitinous chironomids (nonbiting flies), and exoskeletons of copepods and crabs [1]. Interactions of *Vibrio cholerae* with chitin have been implicated in having impacts at different hierarchical scales in the environment, comprising a cellular response (e.g. cell multiplication, chemotaxis, competence), a multicellular response (biofilm formation), a community level interaction (association with chitinous organisms), and a global impact through carbon and nitrogen cycling, and pathogenicity for humans [2]. Consistent with this, nearly all isolates of *Vibrio cholerae* are proficient at degrading chitin (a polymer of composed of chains of β-1,4-linked *N*-acetyl glucosamine (GlcNAc) residues) and utilizing chitin as a carbon and nitrogen source [3, 4]. Expression profiling studies identified three classes of chitin-regulated genes, a subset of which come under control of a membrane-bound chitin-sensing histidine kinase, termed ChiS [3]. These include multiple chitinases that can be used for chitin degradation, the mannose-sensitive hemagglutinin MSHA that contributes to biofilm development, as well as multiple components of an additional pilus called ChiRP which was later identified to be a competence pilus [3, 5]. This prompted Meibom and co-workers to perform transformation experiments with *V. cholerae* grown on different chitin substrates and demonstrate that *V. cholerae* is capable of chitin-induced natural transformation [6].

1.2 Competence and natural transformation in *Vibrio cholerae*

Natural transformation was first described in 1928 by Griffith in *Streptococcus pneumoniae* [7] and since then it has been well-studied in both Gram positive and Gram negative bacteria [8, 9]. Over 80 species of bacteria have been shown to be naturally transformable, including multiple members of the genus *Vibrio*[10]. The majority of work understanding this process in the genus has been with the waterborne, human pathogen *Vibrio cholerae*(see [11] for review).

Many features of the competence machinery and its regulation in *Vibrionaceae* members are similar to systems described for Gram-negative bacteria. Uptake of environmental DNA requires a single Type 4 pilus that is primarily composed of multiple sub-units of PilA. The pilus first binds the DNA at the cell surface and then delivers it through the membrane to the cytoplasm [8, 9, 12]. In *Vibrio cholerae*, the outer membrane secretin pore PilQ allows double-stranded DNA to enter into the periplasm similar to the secretin in *Neisseria* species, such as *Neisseria meningitidis* and *Neisseria gonorrhoeae*[12, 13]. The periplasmic protein ComEA binds the DNA and directs it to the inner-membrane channel ComEC (Figure 1)[12, 14]. One strand of the DNA enters the cytoplasm through ComEC, while the complement strand is degraded by nucleases Figure 1[15]. Once inside the cytoplasm, this DNA may be integrated into the chromosome through homologous recombination (Figure 1). In *Haemophilus influenzae*, the secretin that allows the entry of double-stranded DNA is called ComE; the crucial subunit of the pseudopilus involved in DNA uptake is PilA; and the counterparts of ComEA and ComEC are ComE1 and Rec2 respectively [16-18]. *V. cholerae* possesses homologs of PilQ, PilA, ComEA and ComEC, which play crucial roles in the uptake of

exogenous DNA (Figure 1) [5, 19]. In *N. gonorrhoeae*, PilT protein promotes disassembly of type IV pili and plays a similar role in retraction of the competence pilus (Figure 1). Specifically, *N. gonorrhoeae pilT* mutants produce multiple type IV pili, yet are incapable of pili-dependent phenotypes including natural competence [14]. Similarly *Vibrio cholerae pilT* mutants are deficient for DNA uptake but still produce a competence apparatus[5].

1.3 Regulation of competence and natural transformation in *Vibrio cholerae*

In *V. cholerae* multiple extracellular cues are required to induce several positive regulators of a DNA uptake apparatus. The presence of chitinous material (e.g. zooplankton molts, crab and shrimp shells) promotes expression of the TfoX transcription factor [3, 6]. In the absence of chitin, periplasmic chitin binding protein (CBP) represses ChiS, a membrane bound sensor histidine kinase by direct binding. The presence of chitin oligomers such as $(GlcNAc)_2$ can activate ChiS by binding to CBP and neutralizing its repressive effects on ChiS [20]. Two recent studies also revealed an additional membrane-bound protein TfoS which is a transcriptional factor that can activate transcription of a noncoding small RNA (sRNA) TfoR in response to exogenous $(GlcNAc)_2$. These two studies suggest that binding of $(GlcNAc)_2$, via TfoS, and perhaps with contributions from ChiS, stimulates direct transcriptional activation of TfoR sRNA by the TfoS transcription factor [21, 22]. TfoR directly binds the 5′UTR of the transcriptional regulator TfoX which is a homolog of the *Haemophilus influenza* competence regulator *Sxy* and stimulates translation of the TfoX protein [1, 23]. TfoX up-

regulates multiple genes in the competence apparatus [6].

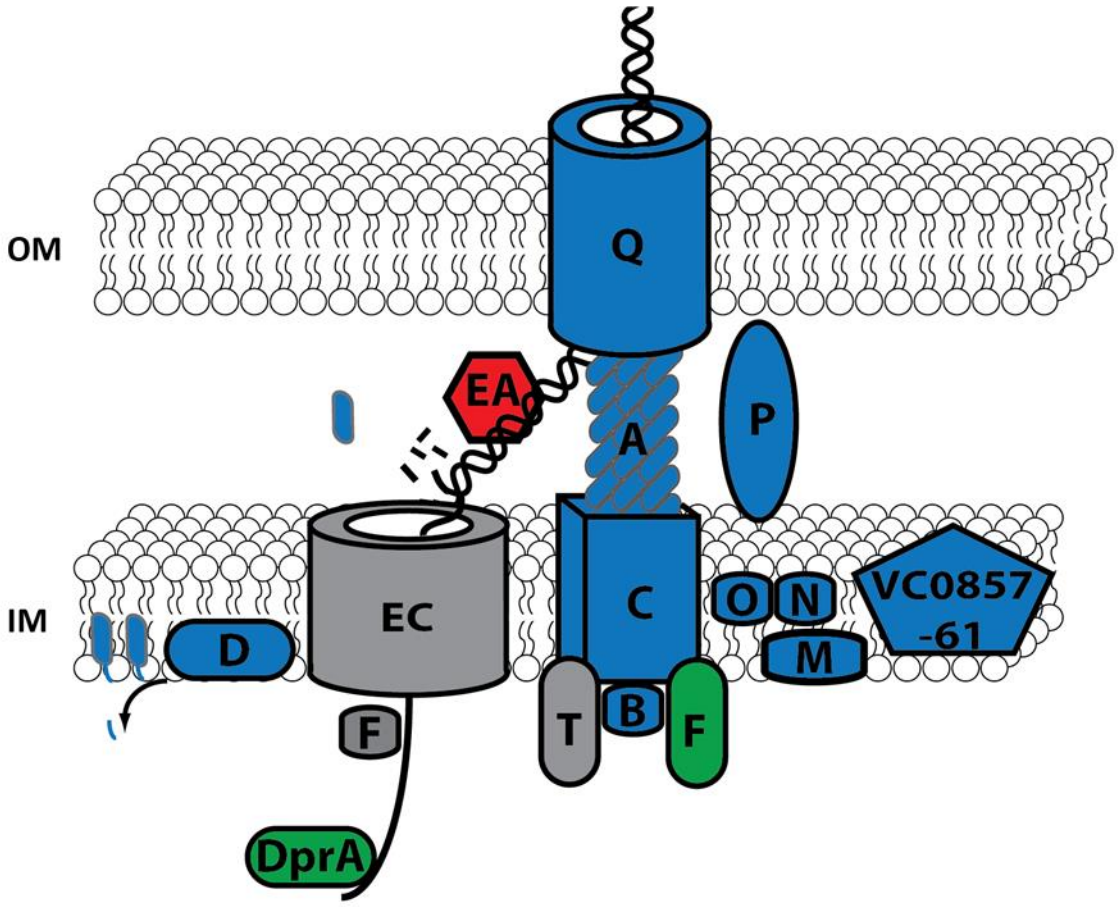


Figure 1. Model of the competence apparatus of *Vibrio cholerae*.

Double-stranded DNA enters the periplasm by means of the secretin pore PilQ located within the outer membrane. The pseudopilus comprised of many repeating subunits of helps shuttle the DNA into the periplasmic space, where it is bound by ComEA, which directs the DNA to the inner-membrane channel ComEC. Other components of a type IV pili system may be involved in this process as well. One strand of the DNA enters the cytoplasm through ComEC, while the complement strand is likely degraded by the periplasmic nuclease Xds. Once internalized, single-stranded DNA is shielded from further nuclease attack by the DNA protecting protein DprA and incorporated into chromosome with the help of recombinase RecA. IM, inner membrane; OM, outer membrane.

Vibrio cholerae cells also express the competence apparatus in response to high cell density. Quorum sensing autoinducer molecules produced by *V. cholerae* cells

accumulate at high density and induce the HapR regulator which is required for DNA uptake and transformation [6]. *V. cholerae* produces two autoinducers, CAI-1 and AI-2, which are released from the bacteria into the extracellular milieu, both of which independently promote DNA uptake. However, CAI-1 which is thought to be a species-specific signal has a greater effect on promoting DNA uptake, indicating that presence of closely related bacteria promotes competence [24]. HapR promotes DNA uptake in multiple ways. First, HapR represses the expression of extracellular nuclease Dns by direct promoter binding [25-27] which prevents degradation of extracellular DNA. HapR also positively regulates the expression of transcriptional regulator QstR via direct promoter binding [25], however induction of TfoX either from a heterologous promoter or in response to chitin is required for QstR expression. Starvation conditions also activate two additional required positive regulators, CRP and CytR [6, 28]. Antonova et al. identified CytR as a positive regulator of competence gene expression and DNA uptake. *V. cholerae cytR* mutants are severely impaired for transcription of *comEA* and for the chitinase gene *chiA-1* [28]. CRP (cAMP receptor protein) is the global regulator of carbon catabolite repression (CCR) in Gram negative bacteria, which together with its allosteric effector cAMP, controls the expression of multiple genes involved in utilization of alternative carbon sources when glucose levels in the cell are low. Expression of DNA uptake genes in *V. cholerae* is subject to CCR, as exogenous glucose prevents transformation and a *crp* mutant is not competent for DNA uptake [6].

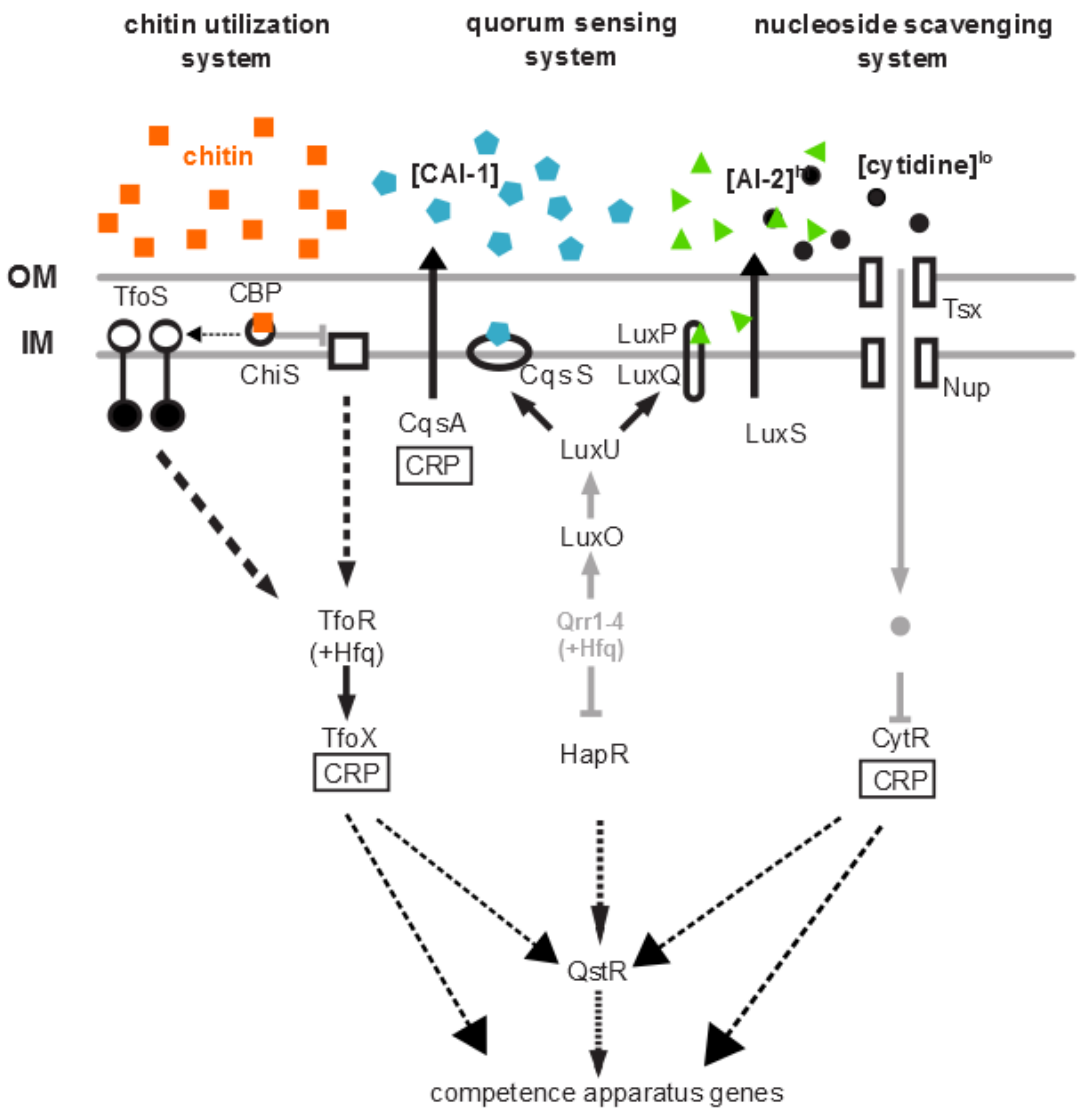


Figure 2. Regulatory network controlling natural competence in *Vibrio cholerae*

Competence expression in *V. cholerae* is regulated by multiple environmental cues such as chitin via TfoX and QstR, Quorum sensing via HapR and QstR and nucleoside starvation via CytR. Starvation also regulates competence for DNA uptake via CRP which is a global regulator and acts at multiple levels. Inputs from the quorum sensing system are discussed in section 1.7.2

CRP is a global regulator that often works in conjunction with other transcriptional regulators to modulate gene expression. *H. influenzae* Sxy, the homolog of TfoX in

Vibrio cholerae, is proposed to direct CRP to interact with a competence regulatory element (CRE) sequence (TGCGA-N6-TCGCA) [29]. Several competence promoters in *Vibrio cholerae* also possess regulatory elements that resemble the CRE regulatory elements [1], however, the exact mechanism of TfoX induction of competence genes is still unknown. One effect of CRP on competence likely also includes modulation of QS since the major autoinducer synthase gene *cqsA* is also CRP controlled.[30]. Similarly, in *E. coli* CytR represses a number of CRP controlled nucleoside metabolism genes such as cytidine deaminase (*cdd*), nucleoside transporters *nupC* and *ompK*, via an anti-activation mechanism through direct binding of these gene promoters. CytR binds via protein–protein interactions with each CRP dimer to a degenerate DNA sequence between the proximal and distal CRP binding sites. [31-36]. While in *E. coli* CytR anti-activates (or represses) genes, in *V. cholerae* genetic evidence supports a model that CytR anti-activation has a positive effect on natural competence [28]. Chapter 2 of this thesis investigates the interplay of these regulators in further detail.

1.4 Measuring Natural Transformation Frequency in *Vibrio cholerae*

1.4.1 Abstract

Many bacteria are genetically programmed to become naturally competent to take up extracellular DNA from the environment via a dedicated uptake apparatus and incorporate the DNA into the chromosome by homologous recombination. This process is called natural transformation, which is distinct from "artificial" transformation coerced by chemical treatment or electroporation. To quantify the frequency of natural transformation, laboratory assays are used that presumably mimic conditions in natural settings, which induce regulatory mechanisms necessary for expression of genes for DNA

uptake and incorporation. Several members of the genus *Vibrio* have recently been shown to become naturally competent. This unit describes assays to measure the frequency of natural transformation in the human pathogen *Vibrio cholerae*. DNA generated as a PCR product or from a genomic DNA preparation serves as the donated genetic material for *V. cholerae* recipient cells. In the basic assay, experimental conditions provide the extracellular signals sufficient for transformation. An alternative assay exploits a heterologous expression system for a critical regulatory factor to enable transformation in the absence of the cognate, natural extracellular signal.

1.4.2 Introduction

Over 80 species of bacteria have been shown to be naturally transformable, including multiple members of the genus *Vibrio*, which are aquatic microorganisms[10]. The majority of work understanding this process in the genus has been with the waterborne, human pathogen *Vibrio cholerae* (see [11] for review). In this bacterium multiple extracellular cues are required to induce several positive regulators of a DNA uptake apparatus. The presence of chitinous material (e.g. zooplankton molts, crab and shrimp shells) promotes expression of the TfoX transcription factor [6]. Prior studies demonstrated that chitinase enzymes produced by *Vibrios* degrade chitin [37], a polymer of N-acetyl glucosamine, into oligosaccharides that serve as an extracellular signal. Quorum sensing autoinducer molecules produced by *V. cholerae* cells accumulate at high density and induce the HapR regulator [38], which triggers QstR expression [25]. Starvation conditions also activate two additional required positive regulators, CRP and CytR [39] [28]. This unit describes three assays for quantifying natural transformation in *V. cholerae*. The assays allow a determination of transformation frequency by measuring

the acquisition of an antibiotic resistance marker into the chromosome of the recipient *V. cholerae* bacteria. The first two assays (Basic Protocol and Alternative Protocol 1) use insoluble chitinous material in medium mimicking the marine environment to activate expression of TfoX and the other positive regulators of competence genes. The third assay (Alterative Protocol 2) uses an allele of the tfoX regulatory gene that obviates the requirement for chitin.

1.4.3 Chitin-induced transformation assay

Many *Vibrio* species have been shown to be naturally transformable. This unit describes the standard assay for quantifying the transformation frequency of *V. cholerae* that is provided naked, extracellular DNA (eDNA) containing a gene for antibiotic resistance (AbR). The first section of the protocol briefly describes the construction of the AbR-marked eDNA. In the detailed, second section, a *V. cholerae* strain that is inoculated into artificial seawater containing a fragment of a chitinous crab shell is then provided the AbR-marked eDNA and incubated for additional time to allow for DNA uptake and incorporation into the chromosome. Transformation frequency is measured by counting on appropriate solid growth medium the number of chitin-associated bacteria that have acquired the AbR marker.

1.4.4 Materials

V. cholerae strain of interest

Luria Broth (LB)

Spectrophotometer

Artificial Sea Water medium (Instant Ocean, cat # SS15-10)]

Shells of blue crab (*Callinectes sapidus*)

12-well microtiter plates; sterile, flat-bottom, standard tissue culture treated plates with lids (Corning Life Sciences DL, cat. # 353043)

Forceps

Appropriate antibiotics

Optional materials

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Promega cat# V3941)

Sterile glass plating beads (Zymo research: cat# S1001)

NOTE: Other insoluble chitinous materials including chitin flakes (Sigma; cat. #C9213) and chitin powder (Sigma; cat. # C7170) are also acceptable chitin sources [40], but clog pipet tips and may give variable results. Soluble GlcNAc oligosaccharide (hexa-N-acetylchitohexaose or $(GlcNAc)_6$) used in prior studies [6] may replace a crab shell fragment, but is costly to obtain in amounts suitable for assays described.

CAUTION: *V. cholerae* is a Biosafety Level 2 (BSL-2) organism. Such organisms pose moderate hazards to laboratory personnel and the environment. When working with these organisms standard microbiological practices must be followed. However, specific training and supervision, personal protective equipment, and laboratory procedures and facilities appropriate for BSL-2 work are also required. See *UNIT xxx* and other pertinent resources (*APPENDIX xxx*) for additional information.

1.4.5 Preparing extracellular DNA (eDNA) and reagents:

The source of eDNA marked with an *abR* gene can be a PCR product, or genomic DNA extracted from a donor *V. cholerae* strain that is isogenic to the recipient except for the presence of an *abR* gene on the chromosome (Figure 3). For example, PCR SOEing [41] may be used to generate a product that carries an *abR* gene flanked by 2 kb of DNA contiguous on the chromosome (Figure 3A). Such a PCR product can be used directly as donor eDNA. Alternatively, genomic DNA can be extracted with a commercial purification kit from a *V. cholerae* donor strain that is isogenic to the recipient (Figure 3B), except for the *abR* gene introduced onto the chromosome prior by allelic exchange [42] or other methods. Alternatively, one can use eDNA carrying a functional allele (*hisD*, for example) that restores prototrophy to an amino acid auxotroph strain (*hisD*$^-$) [6]. The below steps take a total of five days to complete (Table 2: Protocol Time lines).

Ensure sufficient liquid Luria Broth (LB) medium and LB agar plates without and with the appropriate antibiotics are available. Remove the carapace of a cooked blue crab from the rest of the organism and wash with sterile water to remove any debris. Cut up crab shell with scissors into fragments (approximately 1 cm x 1 cm), autoclave, and transfer one fragment to each well of a 12-well microtiter plate with sterile forceps (Figure 4).

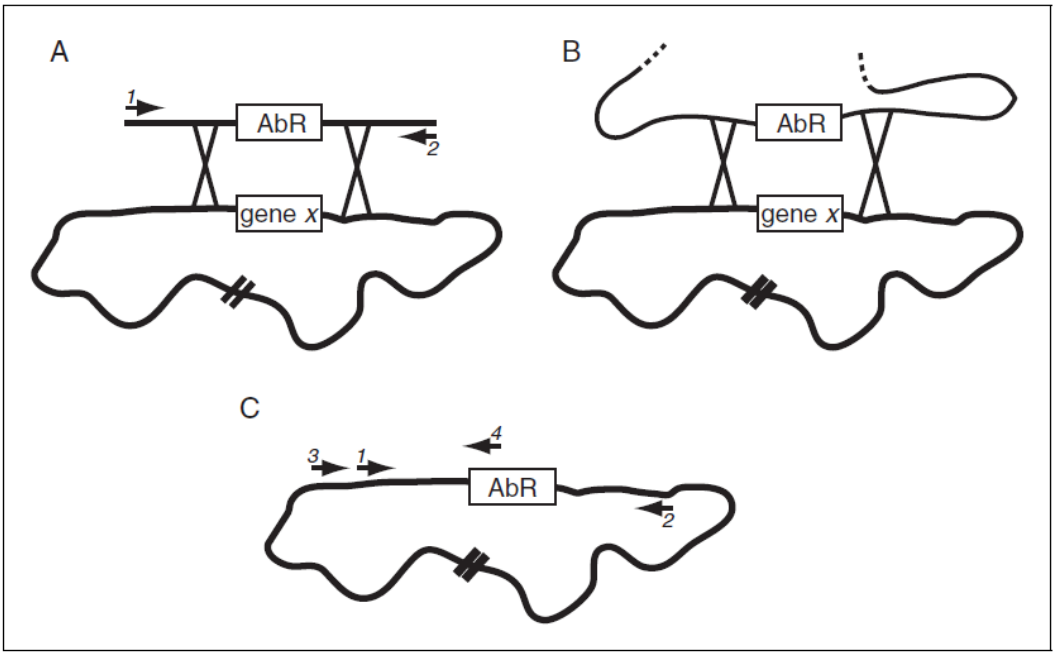


Figure 3. Schematic of transformation with antibiotic resistance marked DNA.

A) a PCR product or B) genomic DNA carrying an antibiotic resistance (*abR*) gene with flanking homology recombines with the recipient *V. cholerae* chromosome (bold) in the cytoplasm after being taken up by the competence apparatus. C) Homologous recombination results in the exchange of the target gene (*geneX*) with the *abR* gene in the chromosome. PCR primers that anneal to the ends on the *abR* gene (arrows 3 and 4) can be used to verify presence of the *abR* gene in putative transformants. However, primers that anneal to regions external to the *abR* gene (arrows 1 and 2) distinguishing between recombination that replaces the target gene and illegitimate recombination events.

1. Inoculate a *V. cholerae* strain of interest into 5 ml of liquid LB medium and incubate aerobically with shaking at 30 °C overnight (~16 hr).

2. Aliquot 70 µL of the overnight culture into 7 ml of fresh LB to dilute 1:100 and incubate for ~90 min to achieve a target OD600 of ~0.3 with a spectrophotometer.

3. Remove an aliquot occasionally to verify the OD600 reading with a spectrophotometer and incubate further if required to reach the desired OD600.

4. Pellet 5 ml of cells from the culture in a centrifuge at 10,000g or higher, and discard supernatant.

5. Re-suspend the pellet in 5 ml of ASW and repeat step 3.

6. Re-suspend the pellet in 10 ml of ASW to achieve an OD600 of ~0.15.

7. Add a 2 ml aliquot of the culture to replicate wells of the 12-well plate containing autoclaved crab-shell fragments (Figure 4), and incubate statically at 30 °C for 24 hr.

8. Typically three replicates wells are used for each strain tested.

9. Remove liquid from each well with a sterile pipet tip and replace with 2ml fresh ASW.

10. Try to preserve the biofilm that accumulates on the crab shell fragment as intact as possible. Not exchanging ASW at this step can lead to lower transformation frequencies as noted in [40].

11. Add 2 µg of AbR-marked eDNA to each well and incubate statically at 30 °C for an additional 24 hr.

12. Transfer each crab shell fragment from a well to a tube with sterilized forceps and suspend in 2 ml of LB.

The tube should be of sufficient size for the crab shell fragment to be fully immersed in LB for vortexing.

13. Vortex extensively for 30 sec to ensure cells attached to each crab shell fragment are sufficiently detached to create a uniform cell suspension.

14. Prepare serial 10-fold dilutions of each cell suspension. In a typical experiment it is sufficient to dilute the cell suspension 1:106 to obtain a reasonable number (~100) of isolated colonies to count on a standard 110 mm petri plate the following day.

15. Spread plate the 10-5 and 10-6 dilutions onto LB agar with no antibiotic.

16. The dilution factors for calculating colony forming units (CFUs) on LB should be empirically determined for each strain tested.

17. Spread plating with sterile glass beads rather than a bent glass rod is recommended when a large number of agar plates (>30) are to be used.

18. Pellet 1 ml of the cell suspension in a micro centrifuge at 10,000g or higher. Discard supernatant and re-suspend cells in 100 µL of LB by pipetting to concentrate 10-fold. Cell suspensions so obtained are designated "concentrated" cell suspensions.

19. Spread plate 100 µL of the undiluted cell suspension and 100 µL of the "concentrated" cell suspension onto separate LB agar with antibiotic.

20. The appropriate dilution scheme for CFU calculation on LB agar with antibiotic should be determined empirically because each strain may transform with a different frequency.

21. Incubate agar plates overnight at 37 °C.

22. A 37 °C incubation here allows for colonies to grow more quickly, but a 30 °C incubation works as well.

23. Count colonies on LB agar to determine the total CFU mL-1 and on LB agar with antibiotic to determine the AbR CFU mL-1, taking into account the dilution factor; and calculate mean transformation frequency given by:

24. Transformation Frequency (T.F.) = $\frac{\#\ of\ AbR\ CFU\ \ mL^{-1}}{\#\ of\ total\ CFU\ mL^{-1}}$

25. Perform verification of true transformants by using an additional marker or by PCR (see Commentary)

Figure 4. A 12-well plate containing sterile Artificial Seawater (ASW) and crab shell fragments.

1.4.6 Alternate protocol 1: Transformation assay with pre-induction by chitin exposure

This alternate protocol reduces the length of the assay one day (Table 2) by initially inoculating the *V. cholerae* strain directly into ASW with a chitin fragment rather than in LB for generating the overnight culture.

1. Inoculate the *V. cholerae* strain of interest into 5 ml of ASW containing a crab shell fragment and incubate aerobically with shaking at 30 °C overnight (~16 hr).
2. Transfer a crab shell fragment with forceps to one well of a 12-well plate containing 2 ml of fresh ASW.

   Typically three replicates wells are used for each strain tested.
3. Add 2 µg of AbR-marked eDNA to each well, and incubate statically at 30 °C for 24 hr.

Shorter incubations times have been described using a similar protocol with chitin flakes rather than crab shell fragments described here [40]

4. Proceed to step 9 of the Basic Protocol.

1.4.7 Alternate protocol 2: chitin-independent transformation assay with a constitutively active allele

The addition of the insoluble crab shell fragment in the Basic Protocol and Alternate Protocol 1 provides the extracellular signal for production of the TfoX regulator, while the accumulation of self-produced quorum sensing molecules at high cell density promotes expression of HapR and the QstR regulator it controls. Although conditions in these two protocols permit induction of *tfoX*, one can also genetically engineer *V. cholerae* strains that express *tfoX* from a heterologous, constitutive promoter [28]. Similarly, strains can be engineered to express *hapR* or *qstR* in a manner that no longer requires high cell density accumulation of extracellular quorum sensing signal molecules [25, 28]. This Alternate Protocol 2 is shorter than the Basic Protocol by one day and also simpler for it eliminates the requirement for ASW and chitin (Table 2). Conditions in LB medium are sufficient to activate the regulatory network controlling transformation in *V. cholerae*, which is under catabolite repression and requires active CRP protein [39].

A protocol with a V. cholerae strain expressing tfoX *constitutively from the chromosome by a non-native promoter is described here (as in[28]). A strain carrying a plasmid-encoded* tfoX *gene under control of an inducible or constitutive plasmid may also be tested [38], but elevated expression levels of tfoX can hinder the growth rate of V. cholerae. See Critical Parameters and Troubleshooting section for additional information.*

Inoculate the *V. cholerae* strain carrying a chromosomally-encoded, constitutive *tfoX* allele into 5 ml of liquid LB medium and incubate aerobically with shaking at 30 °C overnight (~16 hr).

Aliquot 70 µL of the overnight culture into 7 ml of fresh LB medium to dilute 1:100, and incubate for ~90 min to achieve a target OD_{600} of ~0.3 with a spectrophotometer.

Remove an aliquot occasionally to verify the OD_{600} reading with a spectrophotometer and incubate further if required to reach the desired OD_{600}.

Transfer a 2 ml aliquot of the culture to replicate wells of the 12-well plate.

Typically three replicates wells are used for each strain tested.

To each well add 2 µg of AbR-marked eDNA, and incubate statically at 30 °C for 24 hr.

Transfer contents of each well to a micro centrifuge tube, pellet at 10,000g or higher, and discard supernatant.

Re-suspend the pellet in an equivalent volume of LB.

Proceed to step 11 of the Basic Protocol.

Table 1: Typical transformation frequency results

using A) the Basic Protocol or Alternate Protocol 1 with chitin in ASW, or using B) the Alternate protocol 2 with LB.

	Basic Protocol Alternate Protocol 1		Alternate Protocol 2	
Strain	Mean TF	Range	Mean TF	Range
C6706 wild type	3.84×10^{-5}	2.54×10^{-5} to 5.14×10^{-5}	<DL	<DL
C6706 Δ*tfoX*	<DL	<DL	<DL	<DL
C6706 *tfoX*^{constit}	1.36×10^{-4}	6.01×10^{-5} to 2.12×10^{-4}	5.21×10^{-5}	5.09×10^{-5} to 5.33×10^{-5}

Abbreviations: DL, detection limit (no colonies detected on LB agar with antibiotic); TF, transformation frequency.

1.4.8 Commentary

1.4.8.1 Verification of transformed colonies:

It is desirable to confirm that presumptive transformants (colonies counted on the LB agar with antibiotic) are true natural transformants that have the *abR* gene recombined into the chromosome, in contrast to colonies derived from CFUs that acquired spontaneous antibiotic resistance during the assay for other reasons, such as an unlinked mutation. This is particularly important when assaying strains that have not been characterized extensively. The presence of the *abR* gene in the chromosome can be confirmed by PCR. However, external primers that anneal outside of the *abR* gene and not within the marker should be used to distinguish integration at the target locus from illegitimate recombination elsewhere in the chromosome (Figure 3).

Preliminary characterization of the *V. cholerae* strain to be assayed may be useful to exploit the presence of an additional marker (such as the *lacZ* gene) for verifying

acquisition of transforming DNA. For example, the *lacZ* gene encodes β-galactosidase, which catalyzes the degradation of lactose, but also hydrolyzes the synthetic lactose analog, Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), to yield a blue precipitate. If it is determined that the *V. cholerae* strain to be assayed produces Lac^+ (blue) colonies on LB medium containing Xgal because it carries a functional *lacZ* gene, then the eDNA can be designed such that the *abR* gene replaces *lacZ* as the target gene (Figure 3). Transformants can be selected on LB agar supplemented with the appropriate antibiotic and Xgal. While the recipient *V. cholerae* strain is sensitive to the antibiotic and produces blue colonies on Xgal plates, natural transformants will be both antibiotic resistant and display a Lac^- phenotype (white).

1.4.8.2 Transformation assay with a consortium:

The eDNA provided in the laboratory protocols described here is exogenously introduced to a bacterial culture, but transforming DNA in natural settings is derived from other members of the microbial community. Several co-culturing studies have demonstrated that naturally competent *V. cholerae* can incorporate eDNA derived from another *V. cholerae* strain incubated in co-culture on a crab shell fragment [6, 43]. Presumably the eDNA is liberated by cell death and lysis of donor cells in the consortium. When performing the assay in this manner, each *V. cholerae* strain should have a unique chromosomal marker to identify true transformants that carry the chromosomal markers of both the donor and recipient.

1.4.8.3 Genome editing by natural transformation:

One of the most common applications of molecular biology is the construction of defined mutations in organisms of interest to study gene function. This includes creating large mutations (deletions or insertions), modifying regulatory elements such as promoters, or introducing smaller mutations such as single-nucleotide substitutions or point mutations. While this can be achieved using allele exchange vectors, making multiple mutations can be laborious, especially since these methods typically require excision of the selectable marker used in the procedure before further edits can be made [42]. As described here for *V. cholerae*, it is possible to accomplish allelic exchange by natural transformation with PCR products that share homology with flanking regions of a gene of interest while also containing a selectable marker [40]. When making multiple mutations, introducing each sequentially by this method is time-consuming and also limited by the number of antibiotic markers that can be used. A modification of this method allows excision of the antibiotic cassette using flanked FRT sites [44]. However, this requires the presence of a plasmid-encoded Flp recombinase, possibly requiring further curing steps. A relatively rapid and scalable method called MuGENT (Multiplex Genome Editing by Natural Transformation) for simultaneously making multiple scarless edits using PCR products as eDNA template has recently been described [45]. This process relies on the ability of competent *V. cholerae* cells to take up multiple, unlinked PCR products and undergo co-transformation for multiple loci. Thus, the experimenter can select for a single genetic marker like an *abR* gene, and then screen for unlinked mutations introduced by co-transformation by performing PCR for other target loci. When multiple rounds of transformation are required to introduce a large number of edits, one can simply alternate

the selectable markers for each round of MuGENT, potentially making as many edits as desired without being limited by the number of *abR* genes.

1.4.8.4 Competence versus transformation frequency:

The protocols described here allow the experimenter to determine the frequency of successful DNA uptake and incorporation of the *abR* gene. If genomic DNA is the source of eDNA, only a subset of competent cells capable of taking up DNA incorporate the *abR* gene, thus the transformation frequency obtained is likely an underestimate of the number of DNA uptake events or the total number of transformation events. Other recombination events may be occurring that go undetected due to the lack of additional markers. A semi-quantitative method using whole-cell multiplex PCR has been described to distinguish the competence ability of various *V. cholerae* mutants [46]. Another approach described for *Haemophilus influenzae* uses natural transformation to numerically capture the percent fraction of competent cells [47]. Since *Vibrio cholerae* cells can also incorporate unlinked mutations through co-transformation [45], by estimating the transformation frequency of single and co-transformation events, it is also possible to calculate the fraction of competent cells obtained under a given sets of conditions for *V. cholerae* strains.

1.4.8.5 Critical Parameters and Troubleshooting

1.4.8.5.1 Antibiotic selection and testing

When testing new *V. cholerae* isolates for transformation frequency using an *abR* gene as the selectable marker as described in these protocols, it is critical to determine the antibiotic sensitivity of an isolate prior to experimentation. For example, some strains of

V. cholerae display resistance to chloramphenicol due to a chromosomal *cat* gene [48], [49]. As a result, eDNA marked with a gene that confers chloramphenicol resistance is inappropriate for use in a transformation assay because such strains are already resistant to the antibiotic.

A test for the minimum inhibitory concentration or MIC [50] of antibiotic can be used for a *V. cholerae* strain to determine the concentration that prevents growth of visible colonies on LB agar, but permits colony growth for the same strain carrying an *abR* gene on a plasmid or integrated into the chromosome. Once the presumptive MIC is determined it is important to also perform a pilot transformation assay with one or more negative control wells for each new isolate, where AbR-marked eDNA is omitted from each well. The presence of colonies on LB agar with antibiotic derived from such control wells indicates that the antibiotic concentration is insufficient in this assay, as described in more detail below in *Experimental Controls*. With aminoglycoside antibiotics, like kanamycin for example, the MIC can be influenced by changing extracellular conditions [51] like those that occur in assays such as described here with incubation on and in LB and ASW. Thus the results of the pilot experiment can then be used to increase the antibiotic concentration to a level that yields no colonies on LB agar with antibiotic in the negative control and still permits growth when the strain carries the *abR* gene.

1.4.9 TfoX levels

Alternate Protocol 2 described here is appropriate for a *V. cholerae* strain with chromosomally-encoded *tfoX* gene under control of a constitutive promoter (as in [28]). It should be noted that constitutive expression of *tfoX* from a high copy plasmid hinders

growth of *V. cholerae* [39]. If an inducible promoter (such as the IPTG-inducible ptac promoter) controls *tfoX* transcription instead, the inducer (ex: IPTG) concentration should be empirically determined that is sufficient to restore transformation frequency of a *tfoX* deletion mutant but does not impair growth and transformation

1.4.10 Experimental controls

For the assays described here three replicates are strongly recommended for each strain tested, and the use of appropriate controls is critical for interpretation of experimental results. To ensure sterility of media and equipment throughout the experiment, include a "cell-free control" in which chitin containing ASW medium, but no bacteria, is incubated with eDNA and plated identically to other strains. These samples should yield no CFUs on either LB agar or LB agar with antibiotic plates. Likewise, cells incubated without eDNA should also not generate colonies on LB agar containing the selected antibiotic. A known transformation-deficient strain, such as a Δ*tfox* strain is also desirable as an additional negative control that should produce colonies on LB agar but not on LB agar with antibiotic. Finally, the inclusion of a known transformation-proficient strain (such as C6706) [38] that will produce colonies on both LB agar and on LB agar with antibiotic in the assay is also recommended as a positive control

The presence of colonies on LB agar or on LB agar with antibiotics for the "cell-free" control is indicative of a contaminant. Ensure that all equipment and media are sterile throughout the experiment and maintain proper aseptic technique while transferring samples to avoid cross-contamination. If control wells lacking eDNA and wells testing a transformation-deficient strain, such as a Δ*tfoX* deletion mutant, yield colonies on the LB

agar with antibiotics, this is likely due to a contamination problem or indicates that the MIC should be reevaluated. Finally, the failure to detect colonies on the LB agar with antibiotic for a positive control strain, such as C6706, suggests that antibiotic concentrations should be verified. One may also consider repeating the experiment with higher concentrations of eDNA, as some strains of *V. cholerae* produce extracellular DNases that reduce transformation frequency [52]. Finally, one can use an alternative selectable marker, such as a different *abR* gene.

1.4.11 Anticipated Results

The transformation protocols described are useful for determining the capacity of a *V. cholerae* strain of interest to take up and incorporate eDNA into the chromosome. Reference *V. cholerae* strain C6706 has a transformation frequency of ~ 1 x 10^{-5} similar to a strain expressing *tfoX* constitutively, while no transformants are detected in an isogenic mutants with a deletion in *tfoX*. Examples of typical results with the Basic Protocol are shown in Table 1A. The basic protocol described is not only applicable to compare a competence-proficient strain like C6706 to mutants strain (like Δ*tfoX*) derived from it, but also to compare new isolates of *V. cholerae* to a reference strain as well [49].

Transcription of *tfoX* gene under control of a constitutive promoter alleviates the need for chitin to serve as the extracellular signal (Alternate Protocol 2). Table 1B depicts typical results obtained in LB medium with *V. cholerae* using Alternative Protocol 2. In this experimental set-up, no transformants are detected with the C6706 wild type and isogenic Δ*tfoX* strains in the absence of chitin. However, the strain expressing *tfoX* constitutively from a heterologous promoter on the chromosome has a

transformation frequency in LB similar to the C6706 wild type strain incubated with chitin.

Table 2: Protocol Time lines

	Bacterial growth	Bacterial growth	eDNA incubation	Plating bacteria	Counting colonies; verifying transformants	# of days
Basic Protocol	Step 1	Steps 2-6	Steps 7-8	Steps 9-15		5
Alternate Protocol 1	-	Step 1	Step 2	Steps 9-15 of the Basic Protocol		4
Alternate Protocol 2	-	Step 1	Steps 2-4	Step 5-6, and then Steps 11-15 of the Basic Protocol		4

1.4.11.1 Time Considerations

After preparation of eDNA by synthesis of a PCR product or by genomic DNA preparation, the basic protocol takes five days to complete. In contrast, the alternate protocols are four days in duration. A detailed time line for each protocol is provided in Table 2. The amount of time required each day depends on the number of bacterial strains to be tested. The plating of the bacteria and the colony counting are the most time consuming steps. When each strain is tested with triplicate wells, a single 12-well plate is sufficient for analysis of four strains. However, the inclusion of appropriate positive and negative controls (see *Experimental Controls*) will likely require more than a single 12-well plate.

1.5 Type six secretion in *Vibrio cholerae*

Type six secretion systems (T6SS) have been identified in many Gram negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Aeromonas hydrophila* [53] etc. The T6SS in *Vibrio cholerae* was first identified while analyzing transposon mutants of *Vibrio cholerae* strain V52 that were deficient for killing *Dictyostelium discoideum* [54]. Loss of killing activity against *Dictyostelium discoideum* was coupled with loss of secretion of canonical T6SS substrates Hcp (Haemolysin co-regulated protein) and VgrG (Valine-glycine repeat G) [54]. *Vibrio cholerae* has multiple VgrG proteins, but VgrG-1 was shown to have a C-terminal actin crosslinking domain that was cytotoxic to *Dictyostelium discoideum* as well as human macrophage cell lines [54, 55]. Interestingly, VgrG proteins function both as effectors as well as components required for secretion [54], suggesting that they were part of a novel secretion system. Further studies demonstrated that *Vibrio cholerae* could also employ the T6SS to kill other bacterial species such as *E. coli*, *Salmonella typhimurium* and *Citrobacter spp.* as well as non-self *Vibrio cholerae* isolates in a contact-dependent manner [56, 57]. A detailed genetic analysis using defined deletion mutants of genes identified in [54] by Zheng et al [58] revealed the importance of three distinct gene clusters for anti-bacterial as well as anti-eukaryotic activity displayed in Figure 5.

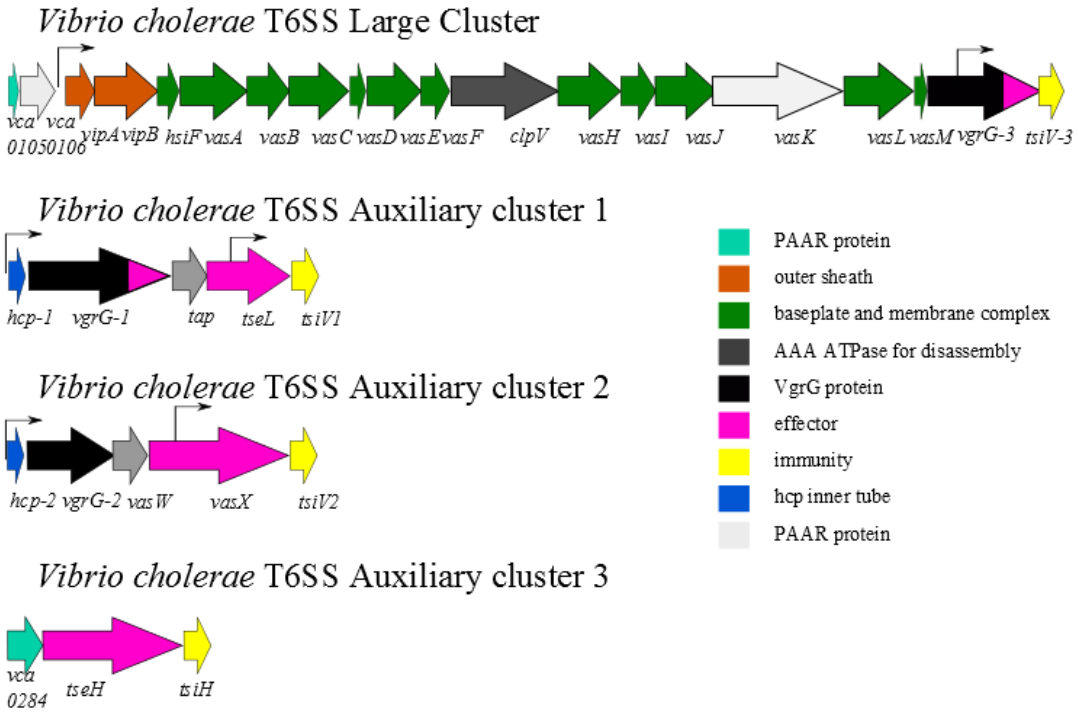


Figure 5. Organization of type six secretion system genes in *Vibrio cholerae* C6706

The large cluster encodes multiple genes that are highly conserved and form a baseplate and membrane complex of the T6SS apparatus in *V. cholerae*. VasK is also a critical structural component and Δ*vasK* mutants do no form functional T6SS apparatus. Auxiliary cluster 1 and 2 both encode identical Hcp proteins that form the inner tube and VgrG proteins that adorn the tip of the T6SS apparatus. Auxiliary cluster 3 has a non-canonical architecture and lacks *hcp* and *vgrG* genes, but like all other T6SS clusters encodes an effector-immunity pair that is secreted by the T6SS apparatus.

A majority of the genes in the Large cluster shown above are required for Hcp secretion, *E. coli* killing as well as toxicity towards *Dictyostelium discoideum* with the exception of VgrG3 which is not required for *Dictyostelium discoideum* killing. PAAR proteins (Proline Alanine-Alanine-aRginine) such as VCA0105 are accessory factors that have been identified as metal-binding, cone shaped proteins that effectively sharpen the β-helical tip of a VgrG trimer and increase effectiveness of T6SS mediated cytotoxicity in other bacterial systems [53]. Additionally, two auxiliary clusters, both coding for Hcp

and VgrG proteins were essential and downstream genes for effectors TseL and VasX were shown to be involved in *E. coli* and *Dictyostelium discoideum* killing [58, 59], but not required for Hcp or VgrG secretion indicating that were substrates for T6SS. Further studies identified TsiV1, TsiV2, and TsiV3 as cognate immunity factors that give protection against the toxic activity of TseL (lipase), VasX (pore forming enzyme) and VgrG3 (a protein with a C-terminal peptidoglycan degrading domain) respectively [60-62]. Interestingly, each cognate immunity protein is expressed from a promoter that is encoded within the coding sequence of the upstream effector [61] see Figure 5, and gives protection against self-intoxication by the upstream effector, or from effectors by T6SS apparatus of sister cells [60, 61]. Indeed, *Vibrio cholerae* isolates harbor diverse effector-immunity pairs [62] that determine compatibility of different *Vibrio cholerae* isolates. While the exact mechanisms of action of immunity proteins are still unknown, presumably most immunity proteins act by mimicking the substrate that each effector targets, as was shown for TsiV3 [63]. Aux cluster 3 was recently identified using a mass spectrometry approach for secreted proteins [64]. TseH encodes for a hydrolase that toxic to *E. coli* while TsiH encodes a cognate immunity factor [64]. This cluster lacks the canonical Hcp and VgrG proteins, but encodes a PAAR domain containing protein VCA0284, upstream of the effector-immunity pair. Lastly, VC1417 (tap) and VasW are DUF4123 containing proteins that are ubiquitous in T6SS in different bacteria [65, 66] are thought to be chaperone proteins that link downstream effectors TseL and VasX to the corresponding VgrG proteins, VgrG1 and VgrG2 respectively and aid in their export [65, 66] via the T6SS apparatus. CHAPTER 3 of this thesis describes the development of

a bioinformatics pipeline used for identifying and classifying novel T6SS proteins in *Vibrio cholerae.*

1.6 Type six secretion structure and mechanism of action

The first glimpse at a T6SS structural component came from electron microscopy and X-ray crystallography of the Hcp1 protein, which is secreted in abundance by *P. aeruginosa* [67] and *Vibrio cholerae* [54]. Its lack of a transport signal sequence, suggested a novel mechanism of protein secretion [68]. Hcp1 was also shown to form a hexameric ring structure [67, 69] that can stack in a head-to-tail fashion to assemble into nanotubes *in vitro*, when disulfide bonds are engineered to stabilize the ring–ring interface [70]. The Hcp1 nanotube structure is similar to those of the bacteriophage T4 tail tube, which functions as a channel for DNA delivery into bacterial cells [71]. Additionally several components of the T6SS machinery resemble bacteriophage components. For example, structure prediction algorithms indicated that VgrG proteins show significant structural homology to a complex called $(gp27)_3$-$(gp5)_3$, which corresponds to the tail spike or "needle" of the T4 phage [53, 72] and can form homotrimeric or heterotrimeric complexes with other VgrGs similar to the T4 phage tail spike protein [71, 72]. VCA0109 has sequence homology to T4 gp25, which forms part of the phage tail baseplate [71] and the *V. cholerae* T6SS proteins VipA and VipB form a tubule complex, similar in structure to the T4 tail-sheath [73, 74]. The VipA/VipB tubule is wide enough to accommodate an 8.5-nm Hcp tube, which could allow VipA/VipB to function like the tail sheath of a contractile phage pushing the Hcp tube through the bacterial outer membrane from the inside. These structural similarities and other biochemical evidence have led researchers to propose that T6SS and phage proteins might have related

evolutionary origins and that the T6SS apparatus may function as an inverted contractile bacteriophage [53, 72, 75] . The current model of T6SS mechanism of action is largely based on microscopy studies visualizing fluorescent VipA-GFP fusion proteins that show a large VipA-containing sheath structure exists inside cells and undergoes cycles of extension, contraction, disassembly, and reassembly (Figure 6). The T6SS sheath polymerizes from a membrane-bound complex in an extended conformation, and like phage, the extended sheath structure then undergoes a rapid contraction event, estimated to occur in less than 5 ms [76] and helps deliver toxic effectors into neighboring cells (Figure 6). Disassembly of the contracted sheath structure is driven by the AAA-ATPase ClpV, which recognizes only the contracted form of the T6SS sheath in both *V. cholerae* and *P. aeruginosa* [76, 77].

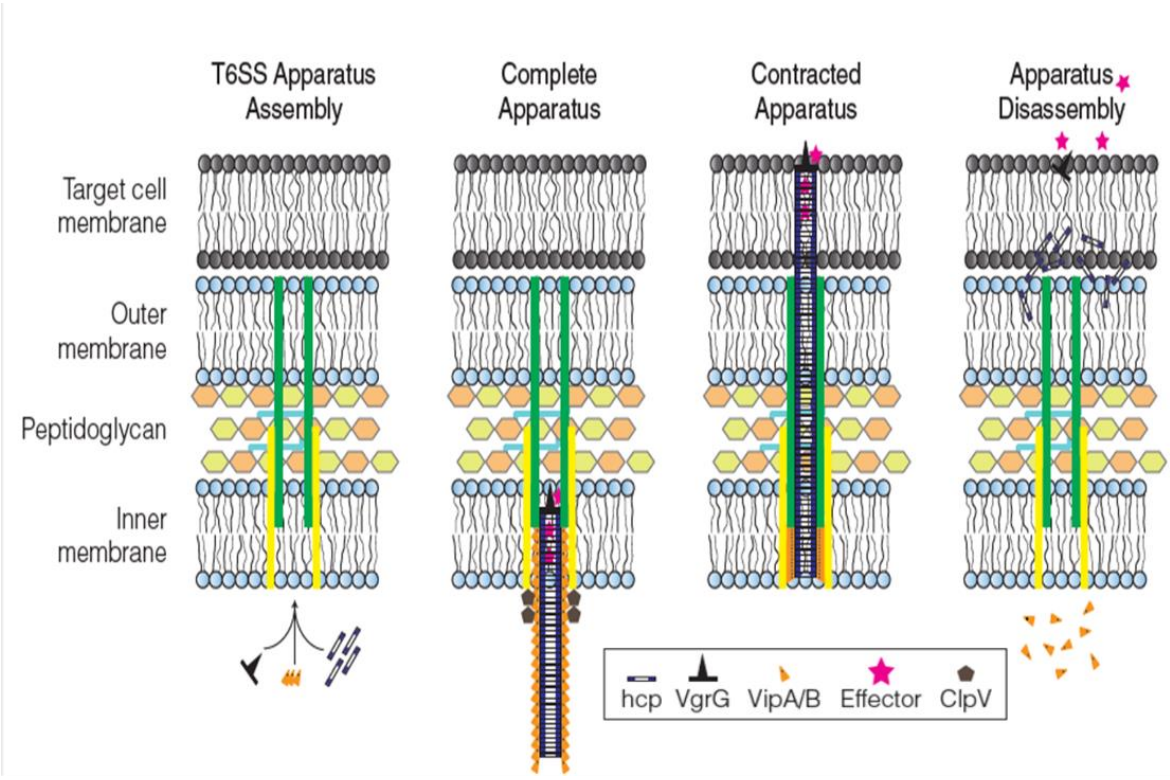


Figure 6. Type six secretion system mechanism of action

Type six secretion assembly is initiated with VgrG proteins assembling at the tip of the needle complex with baseplate components in the inner membrane. An Inner Hcp tube covered by an outer sheath of VipA/VipB proteins is assembled inside the cytoplasm. A sudden (~5 ms) contraction event releases the T6SS needle and penetrates neighboring cells and delivers toxic effectors using several different mechanisms described below. Apparatus disassembly requires the action of ATPase ClpV.

As many as five distinct mechanisms of effector translocation have been proposed in the multiple effector translocation VgrG (MERV) model [53]. First, VgrG proteins which form the "spike" of the T6SS apparatus and are essential for assembly, can themselves encode variable C-terminal anti-eukaryotic and anti-bacterial effector domains, such as the actin cross-linking domain from VgrG-1 and the lysozyme-like domain of VgrG-3 [60, 72]. In *V. cholerae*, VgrG-2 is required along with at least one of VgrG-1 and VgrG-3 [58], suggesting that formation of hetero-trimeric complexes of VgrG-2 and VgrG-1 or VgrG-2 and VgrG-3 is required for T6SS assembly. Second, DUF4123 containing Type six secretion adaptor proteins (Taps) cross-link specific effectors e.g. TseL, to cognate VgrGs (VgrG-1). Deletion of the adaptor proteins allows for a functional T6SS assembly, but prevents translocation of the specific effector protein [65, 66]. Third, PAAR proteins (Proline Alanine-Alanine-aRginine) have been known to carry variable effector motifs [78] , however in *Vibrio cholerae,* PAAR proteins have not been shown to be required for T6SS apparatus assembly or to have specific effector activities. Two additional mechanisms have been proposed, but lack functional validation [53]. Like VgrG, PAAR proteins can also have additional extension domains with various predicted effector activities, suggesting that the hundreds of PAAR proteins identifiable in genome databases may be T6SS effectors as well [78]. Because the interaction between PAAR proteins and the C-terminal end of the VgrG trimer is driven by hydrogen bonding between the backbones of the respective proteins, it has been proposed that various

PAAR proteins might be able to bind to any given VgrG trimer [78]. Lastly, the inner Hcp tube might also function as a passive translocation channel [53], however this mechanism is likely restricted to small effector proteins since the inner diameter of the Hcp tube is only about 40 Å wide.

1.7 Type six secretion system regulation in *Vibrio cholerae*

The initial transposon screen in strain V52 that identified the T6SS components in *Vibrio cholerae* also showed that mutant containing transposon insertions in genes encoding the alternative sigma factor RpoN and an internal transcriptional regulator VasH, which is encoded in the large T6SS cluster (VCA0117), were impaired for T6SS function [54]. The role of these two transcriptional factors was further explored [79-81] and both VasH and RpoN were shown to coordinately up-regulate transcription of Auxiliary clusters 1 and 2 by direct binding to the *hcp* gene promoters in both operons. However, the initial discovery of T6SS in *V. cholerae* was made using a strain that constitutively expresses this system under laboratory conditions i.e. V52, while clinical isolates such as N16961 do not [54]. Consistent with this pattern, a recent survey found that a majority of clinical *Vibrio cholerae* isolates could not constitutively kill *E. coli* prey, while a majority of the environmental isolates were proficient for T6SS dependent killing [4]. However, several studies have noted a wide range of environmental cues that are required to activate T6SS expression and assembly in diverse *Vibrio cholerae* isolates [53, 82-85] in which T6SS expression is not constitutive. This diversity of regulatory strategies termed "pathoadaptivity", may be indicative of evolutionary adaptations that are advantageous to the niche occupied by specific isolates. So far, all studied isolates belonging to the O1 and O139 serogroups such as clinical isolates C6706 and A1552 tightly control

expression of the T6SS under laboratory conditions and need to be induced by environmental signals that are both biotic and abiotic in nature [82, 86]. CHAPTER 2 of this thesis explores the complexities of this regulation in further detail, but a brief overview is presented here.

1.7.1 Environmental stress

In a study by Ishikawa et. al, *Vibrio cholerae* A1552 was shown to secrete Hcp in response to several environmental cues such as elevated temperature and high salinity and high osmolarity [82]. The activation of the T6SS in response to elevated temperature appears to be regulated in part by the cold shock protein CspV. Deletion of *cspV,* significantly decreases the transcription of *hcp*, resulting in less killing of bacterial prey at 25°C and 37°C [82, 87]. Regulation of T6SS in response to high osmolarity conditions was dependent on the osmolarity regulator OscR and mutants lacking OscR were proficient for Hcp secretion [82], however the mechanism by which this operates was not elucidated. Similarly carbon catabolite repression mediated by the CCR protein CRP was also shown to promote Hcp secretion in response to starvation conditions [88], while indole production that often accompanies stationary phase growth also promotes expression of T6SS genes [89]. Interestingly, deletion of motility regulators such as *fliC, flrA* and *flrC* promotes T6SS in *V. cholerae* strains suggesting a role for T6SS during times when *Vibrio cholerae* adopts a sessile life-style in surface attached biofilms[90].

1.7.2 Quorum sensing and Chitin induction

Quorum sensing (QS) is a form of bacterial communication that occurs through the production, secretion, and sensing of small molecules known as auto-inducers [91, 92].

This communication allows alterations in gene expression to occur across a population of bacteria in response to changing cell density, which is signaled by increasing levels of auto-inducers. QS is known to regulate a variety of behaviors important for the aquatic and intestinal life cycles of *V. cholerae* including biofilm formation, motility, natural competence, and virulence factor production [6, 93, 94]. In *V. cholerae,* QS mediated gene regulation occurs through a phosphorelay cascade modulated by four sensor histidine kinases, CqsS, LuxPQ, CqsR, and VpsS. CqsS and LuxPQ sense the levels of cholera autoinducer 1 (CAI-1) and autoinducer 2 (AI-2), respectively, while the ligands for CqsR and VpsS have not been identified. At LCD, these four histidine kinases phosphorylate the phosphotransfer protein LuxU, which in turn phosphorylates LuxO. Phosphorylated LuxO activates the expression of four small RNAs known as Qrr1-4, which bind to and destabilize the mRNA transcripts of the transcriptional regulator HapR. At HCD, however, LuxO is unphosphorylated, and transcription of *qrr1-4* is inactive, thus permitting the translation of the HapR mRNA, which in turn regulates multiple phenotypes [92, 95-98]. Quorum sensing control of T6SS genes in *Vibrio cholerae* occurs at multiple levels. First, LuxO represses T6SS expression [86] in *Vibrio cholerae* C6706 and *ΔluxO* strain is proficient for Hcp secretion, VgrG translocation into the extracellular milieu as well as increased hemorrhage in the intestinal lining of rabbit hosts. Similar results were later obtained for *V. cholerae* A1552 [82]. Deletion of *tsrA* enhances these phenotypes in a *ΔluxO* C6706 strain, but the mechanism for this is unclear [86]. Further studies revealed that the Qrrs repressed expression of T6SS large cluster by direct binding to VCA0107 mRNA, which is the first gene in the T6SS large cluster operon [97] that in turn represses expression of Auxiliary clusters 1 and 2 through VasH.

Similarly, activation of HapR at high cell density leads to up-regulation of the T6SS machinery [82, 97] . Further evidence for HapR control of T6SS was obtained in RNA sequencing studies of *V. cholerae* on chitin [84], see chapter 2 for more details. HapR is also required for the expression of transcriptional regulator QstR [25] which was recently shown to be involved in activation of T6SS [84]. QstR expression is contingent upon induction of TfoX, which is up regulated in the presence of chitin. All three regulators HapR, QstR and TfoX are required to induce T6SS system expression and inter-bacterial killing on chitin in C6706 and A1552 [25, 84], (chapter 2). These adaptations might be critical in shaping survival of *Vibrio cholerae* cells on chitin surfaces in the marine environment. Recently, transcriptional regulator TfoY which is a TfoX homolog was implicated in regulating T6SS system genes in a TfoX and QstR independent manner including in V52 suggesting that different regulatory schemes might govern T6SS for defense against predatory amoebae [83]

1.7.3 Type six secretion in the host

The ubiquity of Type six secretion system genes in the human pathogen *V. cholerae* have prompted researchers to speculate that T6SS might play important roles in host colonization by competing with the host microflora and killing active macrophages [99]. Using the infant mouse model, Ma, et. al showed that T6SS of *V. cholerae* V52 was responsible for actin cross-linking of host cells and led to increased inflammation and diarrheal response [100]. However, as noted earlier, most clinical isolates do not have a constitutively active T6SS. Interestingly, a Tn-seq study designed to identify genes critical in survival inside the host using clinical isolate C6706 discovered greatly reduced survival of a C6706 mutant lacking the T6SS immunity protein Tsiv3 inside an infant

rabbit intestine [101]. Competition experiments revealed that this decrease in survival was dependent on the presence of T6SS^{+} sister cells which were able to kill susceptible *tsiv3* cells, indicating that T6SS genes might be activated using host factors. Additional work showed that the *V. cholerae* T6SS is also responsive to host signals, such as mucin and bile salts. [102] Mucins, the main component of the mucus layer in the intestine, are known to increase T6SS-mediated killing of bacterial prey, while the bile salt deoxycholic acid represses T6SS killing via inhibition of T6SS tube formation. The production of deoxycholic acid is facilitated by the commensal bacterium *Bifidobacterium bifidium*, which is capable of metabolizing certain bile acids to deoxycholic acid [102]. Thus, *Vibrio cholerae* might utilize T6SS weaponry to effectively compete in the host environment.

CHAPTER 2. CytR is a global positive regulator of competence, type VI secretion and chitinases in *Vibrio cholerae*

2.1 Abstract

The facultative pathogen *Vibrio cholerae* transitions between its human host and aquatic reservoirs where it colonizes chitinous surfaces. Growth on chitin induces expression of chitin utilization genes, genes involved in DNA uptake by natural transformation, and a type VI secretion system that allows contact-dependent killing of neighboring bacteria. We have previously shown that the transcription factor CytR, thought to primarily regulate the pyrimidine nucleoside scavenging response, is required for natural competence in *V. cholerae*. Through high-throughput RNA sequencing (RNA-seq), we show that CytR positively regulates the majority of competence genes, the three type VI secretion operons, and the four known or predicted chitinases. We used transcriptional reporters and phenotypic analysis to determine the individual contributions of quorum sensing, which is controlled by the transcription factors HapR and QstR; chitin utilization that is mediated by TfoX; and pyrimidine starvation that is orchestrated by CytR, toward each of these processes. We find that in *V. cholerae*, CytR is a global regulator of multiple behaviors affecting fitness and adaptability in the environment.

2.2 Author Summary

Studies in *Escherichia coli* identified CytR as a specialized repressor of a modest number of genes used exclusively for scavenging and metabolizing nucleosides. Our prior work

revealed CytR acted positively on several genes for natural competence in *Vibrio cholerae*. Here we discover an expanded regulatory role for CytR in this important human pathogen. Specifically we demonstrate that CytR governs not only the small set of nucleoside scavenging factors, but also a larger repertoire of behaviors including natural competence, chitin utilization, and type VI secretion, which are tailored for the aquatic niche of this waterborne bacterium.

2.3 Introduction

Vibrio cholerae is the causative agent of the diarrheal disease cholera and occupies a range of freshwater and marine environments. The bacterium has been found in association with plants, algae, cyanobacteria, fish, and marine and freshwater invertebrates [103] and its attachment to copepods has been implicated in disease transmission [104]. *Vibrios* and other chitinolytic bacteria degrade the chitinous surfaces of copepods and zooplankton to soluble (GlcNAc)n oligosaccharides that are then imported and utilized as a carbon source [105].

When *V. cholerae* associates with chitin, in addition to chitin utilization enzymes, it also produces a DNA uptake apparatus for natural transformation [6]. Components of this apparatus include a pilus that extends into the extracellular environment as well as inner and outer membrane channels that transport DNA molecules into the cytoplasm where it can recombine, allowing horizontal gene transfer [5, 106]. It was recently discovered that in *V. cholerae*, growth on chitin induces expression of the Type VI secretion system (T6SS), an apparatus that penetrates and delivers toxic effectors into the cytoplasm of

neighboring cells, causing contact-dependent lysis [53]. Lysed cells liberate DNA that can then be used for natural transformation [84].

Genes of the chitin utilization program, natural transformation, and the Type VI secretion system are under the control of a common regulator TfoX, induced by growth on chitin [6]. TfoX is post transcriptionally activated by the TfoR sRNA in response to GlcNAc oligomers liberated from chitinous material [105, 107], but the means by which TfoX activates its downstream targets is poorly understood. A current model suggests that *Haemophilus influenzae* Sxy, a TfoX homolog, may directly activate competence gene promoters by interaction with the cAMP receptor protein (CRP) [108, 109]. However, direct binding of TfoX to its putative target promoters in *V. cholerae* has not been demonstrated.

Quorum sensing at high cell density, mediated by the regulator HapR, is also required for natural transformation and Type VI secretion in *V. cholerae* in response to secreted autoinducer signals at high density[6, 84, 110, 111]. HapR accumulation down-regulates transcription of the gene for a secreted deoxyribonuclease (*dns*) via direct promoter binding, facilitating DNA uptake by reducing extracellular DNA degradation [112, 113]. HapR also directly activates transcription of the gene coding QstR, a transcriptional regulator that positively controls expression of the periplasmic DNA binding protein *comEA* in the presence of TfoX and the three gene clusters encoding the Type VI secretion system [84, 113]. The mechanism by which QstR activates its target genes is not known, but may require a putative co-factor [109, 113].

We previously identified another regulator, CytR, which positively regulates competence by transcriptional activation of two genes, *comEA* and *pilA*, and upregulates chitin utilization by activation of the chitinase gene *chiA-1* [114]. In *Escherichia coli,* the cytidine repressor CytR negatively regulates a small set of nucleoside scavenging and metabolism genes, including *udp, cdd, ompK (tsx),* and *cytR* itself via a CRP-dependent anti-activation mechanism [115]. CRP binding sites in the *udp* promoter of *E. coli* allow transcriptional activation by recruitment of RNA polymerase (RNAP) [116, 117]; but specific spacing of two DNA-bound CRP dimers also stabilizes weak CytR-DNA binding interactions that inhibit RNAP recruitment. *V. cholerae* CytR represses *udp* transcription in *V. cholerae* and in an *E. coli cytR* deletion mutant [114, 118]. Thus, CytR in *V. cholerae* behaves as a negative regulator of the *udp* nucleoside scavenging gene, as in *E. coli*, and also serves as a positive regulator of one chitinase and two competence genes.

Here we show that CytR, like TfoX and HapR, is a global regulator in *V. cholerae*. In addition to repressing multiple nucleoside scavenging and metabolism genes, transcriptome analyses demonstrate that CytR also positively regulates the majority of known competence genes, the three known Type VI secretion system (T6SS) gene clusters, and four chitinase genes in *V. cholerae*. Distinct regulatory patterns reveal that the specific mechanism of regulation and the participation of each transcription factor differ for each of the three phenotypes studied.

2.4 Materials and Methods

2.4.1 Bacterial strains, plasmids, and culture conditions.

All *V. cholerae* strains were derivatives of a streptomycin resistant C6706 El Tor biotype O1 strain (BH1514), and all *E. coli* strains were derivatives of MG1655 and are described in detail in (

Table 5). Bacteria were commonly grown at 37°C in Luria broth (LB) under constant shaking, or statically on petri plates containing LB agar, supplemented with ampicillin (100 µg/mL), kanamycin (50 µg/mL), chloramphenicol (10 µg/mL for *V. cholerae* and 25 µg/mL for *E.coli*), diaminopimelic acid (DAP 50 µg/mL), and streptomycin (5 mg/mL) where appropriate.

2.4.2 Construction of genetically modified strains of *Vibrio cholerae*.

In-frame deletions and promoter-replacement mutants in *V. cholerae* were constructed by allelic exchange using pKAS32-based plasmids [42] indicated in

Table 5.

2.4.3 Recombinant DNA techniques.

Standard molecular biology-based methods were utilized for DNA manipulations. DNA modifying enzymes and restriction nucleases (Promega and New England Biolabs), Gibson assembly mix (New England Biolabs), Phusion DNA Polymerase (New England Biolabs), and Taq DNA polymerase (Promega) were used following the manufacturer's instructions. All modified DNA fragments were tested by colony PCR and verified by Sanger sequencing (Eurofins).

2.4.4 Transformation assays.

Transformation assays in LB medium were performed as described [119]. Briefly, triplicate *V. cholerae* cultures grown overnight in LB medium were pelleted using centrifugation and resuspended in fresh LB to an OD_{600} of ~0.1. Diluted cultures were grown until an OD_{600} of ~ 0.3 was reached, and genomic DNA marked with a kanamycin resistance cassette [110] was then added at a final conc. of 1 µg/mL. Cultures were incubated at 30°C for 24 hours and transformants were assayed by plating on LB agar plates with and without kanamycin. Transformation frequency was defined as Kan^R cfu mL^{-1}/total cfu mL^{-1}.

2.4.5 RNA-Sequencing.

Total RNA from 3 independent cultures of four *V. cholerae* strains grown to exponential phase (OD_{600} 0.5-0.7) in LB medium at 37°C was extracted using mirVana miRNA isolation kit (Ambion). DNAse treatment for removal of genomic DNA was performed using TURBO DNA free kit (Ambion). Detection of contaminating genomic DNA was carried out by performing PCR amplification with primers specific for 16S rRNA loci, and DNAse treatment was repeated until no PCR products were detectable. DNA-free total RNA samples were purified using RNEasy Minelute kit (Qiagen). All kits were used as per manufacturers' instructions unless described otherwise.

Further processing of the samples was conducted by Eurofins (Louisville, USA) using a standardized Illumina RNA Sequencing pipeline. Briefly, RNA sample quality was determined using an Agilent 2100 Bioanalyzer and Qubit, before ribodepletion using a Ribo-Zero Magnetic kit (Epicentre) for Gram-negative bacteria. Sequencing was performed using a HiSeq2000 sequencer (Illumina) and 100bp paired end reads were obtained.

Reads were mapped to chromosomes I and II of *V. cholerae* N16961 (European nucleotide archive accession numbers AE003852.1and AE003853.1) using Bowtie2 [120]. Mapped reads were visualized using Seqmonk v2.8 (Babraham Bioinformatics) and read counts obtained using Seqmonk's RNA-seq Quantitation pipeline. Statistical analysis for differentially expressed genes was performed using the DESeq package [121] and genes with > 2 fold change and p-value <0.05 were analyzed. For the RNA-seq statistics and entire list of differentially expressed genes, see **Table 4** and **Table 6**. Heat maps were generated using R statistical package (v 3.0.2). [122]

2.4.6 Bioluminescence Assays.

V. cholerae strains carrying lux-based reporter plasmids were grown on LB agar plates containing chloramphenicol at 37°C overnight. Cells were resuspended in LB medium containing chloramphenicol to an initial OD_{600} of 0.01 and incubated with shaking at 37°C until an OD_{600} of 0.8-1.0 was reached. Bioluminescence and absorbance were quantified as described previously [96]. Bioluminescence was measured using a Wallac model 1409 liquid scintillation counter as described previously [123] and optical density of each culture was measured with a spectrophotometer. Relative Light Units (RLU) are defined as counts $min^{-1} mL^{-1}/OD_{600}$. Single-time-point experiments were performed in triplicate.

2.4.7 T6SS killing assay.

The T6SS killing assay was modified from previously described methods [56]. *V. cholerae* and *E. coli* strains grown overnight on LB plates at 37°C were resuspended in LB medium to an OD600 of 0.01 and incubated with shaking at 30°C until they reached an OD600 of 1.0. Predator and prey strains were mixed at a ratio of 10:1 and 50 µL of each suspension was spotted onto sterile Whatman cellulose gridded filters (GE Healthcare) placed on LB plates. After incubation at 37°C for 3 hours, filters were removed and washed with 5 mL LB medium to recover cells. Dilutions of the cell suspension were plated on LB agar supplemented with chloramphenicol to determine counts of surviving prey.

2.4.8 Chitinase plate assay.

Colloidal chitin was prepared from practical grade chitin (Sigma) derived from shrimp shells as previously described [124, 125]. Colloidal chitin plates were made by mixing 2% w/v colloidal chitin with LB medium buffered to pH 7.0 with 0.1M phosphate buffer. Strains were incubated overnight at 37°C in LB broth, diluted to an OD_{600} of 1.0 and 10 µL of each suspension was stabbed into the chitin agar. The plates were incubated at 37°C for 72 hours and the zone of chitin clearing for each colony was recorded.

2.5 Results

2.5.1 CytR is a global regulator in *Vibrio cholerae*

In *Escherichia coli*, the cytidine repressor CytR negatively regulates a small set of pyrimidine nucleoside scavenging and metabolism genes, including uridine dephosphorylase, *udp* [115]. In *Vibrio cholerae* (El Tor strain C6706), we recently demonstrated that in addition to repressing *udp*, CytR also positively regulates competence genes *comEA* and *pilA*, and the chitinase gene *chiA-1* [114]. We now find that CytR is required for expression of the majority of known competence genes, the three Type VI secretion system (T6SS) clusters, and four known chitinase genes (Figure 8B).

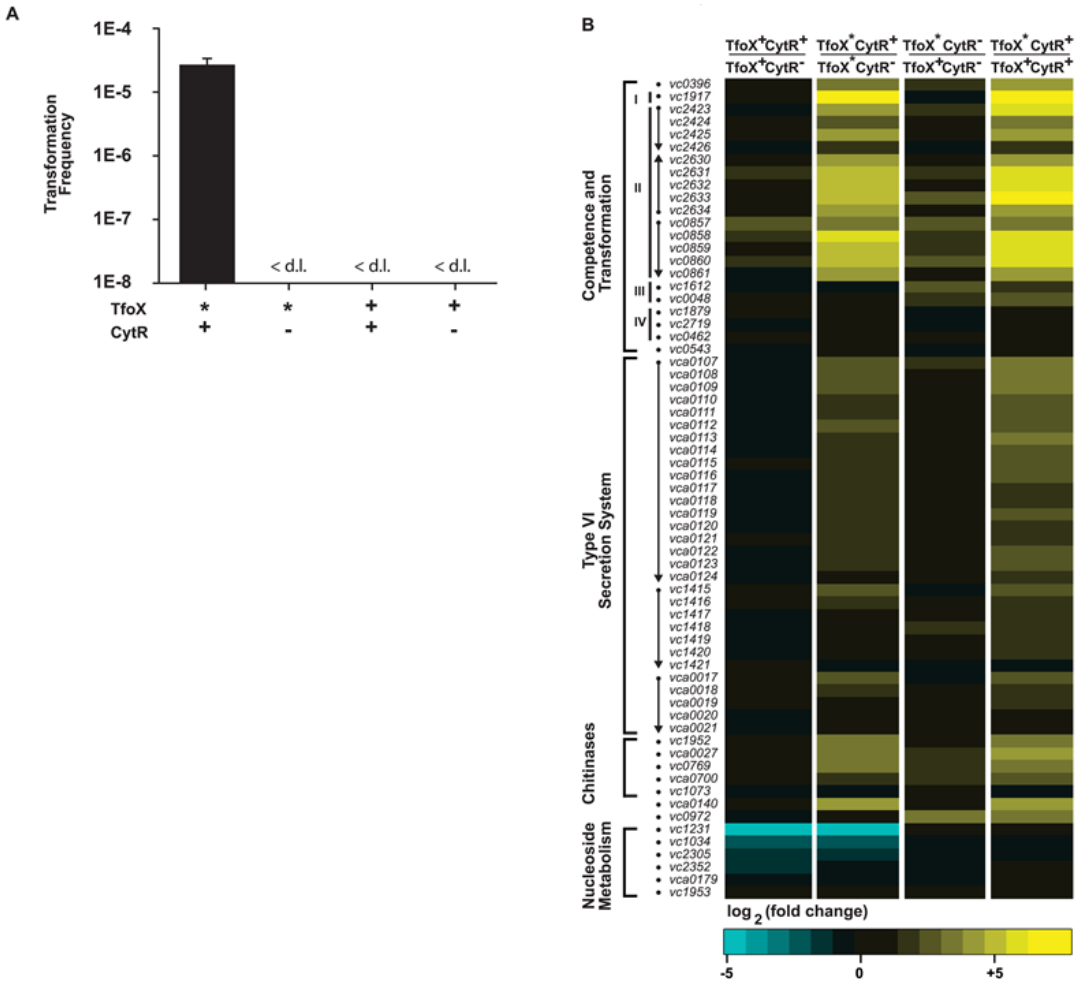


Figure 7: CytR and TfoX co-regulate natural competence, chitinase expression and the type VI secretion system.

Panel A: *V. cholerae* C6706 is capable of natural transformation in LB medium lacking chitin if *tfoX* is constitutively expressed (TfoX*, bar 1) but not if *tfoX* is under control of its native promoter ($TfoX^+$, bars 3 and 4). No transformants were detected in the absence of CytR ($CytR^-$, bars 2 and 4). Transformation frequency is expressed as the number of kanamycin resistant cfu mL^{-1} divided by total cfu mL^{-1}. The limit of detection (d.l.) is 1 x 10^{-8}. Data are shown as mean ± standard deviation from three independent biological replicates. Panel B: Heat map of genes differentially regulated by CytR in the absence ($TfoX^+$, column 1) or presence (TfoX*, column 2) of TfoX induction, and genes differentially regulated by TfoX in the absence ($CytR^-$, column 3) or presence ($CytR^+$, column 4) of a functional *cytR* gene. The majority of known competence genes are positively regulated by both TfoX and CytR and can be classified into four distinct regulatory classes (see text for details). CytR and TfoX positively regulate the three

known T6SS gene clusters as well as four chitinase genes. CytR negatively regulates nucleoside uptake and catabolism genes in a TfoX-independent manner.

In our prior study, CytR-mediated regulation was measured in a strain with a *luxO* deletion (Δ*luxO*) and a chromosomal *tfoX* allele (*tfoX**) under control of a heterologous *ptac* promoter, constitutively expressed due to the lack of a functional LacI repressor in *V. cholerae* C6706. The strain is also merodiploid for *hapR* (an additional copy of *hapR* under its native promoter is integrated at the *lacZ* locus) to avoid bypass suppressor mutations in *hapR*, which often occur in lab settings and render C6706 deficient in HapR-dependent quorum sensing [94]. In LB medium this Δ*luxO* Δ*lacZ:hapR tfoX** strain (EA349) expresses both TfoX and HapR independent of chitin or high cell density, respectively [114]. As a result, EA349 (denoted here as $TfoX^{*}$ $CytR^{+}$) is transformable in LB, whereas its isogenic Δ*luxO* Δ*lacZ:hapR tfoX** Δ*cytR* mutant ($TfoX^{*}$ $CytR^{-}$) is not (Figure 8A). A Δ*luxO* Δ*lacZ:hapR* strain that carries the native *tfoX* allele (denoted here as $TfoX^{+}$ $CytR^{+}$) and an isogenic Δ*luxO* Δ*lacZ:hapR* Δ*cytR* strain ($TfoX^{+}$ $CytR^{-}$) are also not transformable with antibiotic-marked linear DNA because the native *tfoX* allele is poorly expressed in the absence of chitin. Thus transformation in LB medium requires the *tfoX** allele ($TfoX^{*}$) and the presence of the native *cytR* allele ($CytR^{+}$).

2.5.2 *Transcriptome analysis.*

To define the set of genes regulated by CytR in *V. cholerae* we performed high throughput RNA sequencing (RNA-seq) on triplicate samples of each of these four strains ($TfoX^{*/+}$ $CytR^{+/-}$) grown to mid-log phase (OD_{600} of 0.5-0.7) in LB medium. In total, 12 RNA samples were sequenced, generating over 216 million 100 bp paired end reads, which were then mapped to the reference genome (El Tor *V. cholerae* N16961)

and read counts were obtained. Relative abundance of each transcript was determined by applying the RPKM correction to the read counts and pairwise comparisons between different strains were used to calculate fold changes in gene expression [126]. Data were analyzed for genes with transcript abundance differences $\geq$ 2-fold. Replicate samples showed a high degree of correlation (R>98%).

2.5.3 CytR negatively regulates nucleoside metabolism in *Vibrio cholerae*.

To measure the effect of CytR in the absence of TfoX induction, transcript abundance of the TfoX+ CytR+ strain (carrying the native tfoX allele) was compared to the isogenic TfoX+ CytR- mutant. Increased expression of 23 genes and decreased expression of 29 genes was observed in the TfoX+ CytR+ strain (Table 6). Consistent with prior studies [114, 118], the set of CytR-repressed genes included udp (vc1034) (Figure 8B column 1 and 2, blue). Negative regulation was also observed for *E. coli* homologs of additional nucleoside metabolism genes including: cytidine deaminase, cdd (vc1231); outer membrane nucleoside transporter, ompK (vc2305); and one of three genes annotated in *V. cholerae* as putative inner membrane nucleoside uptake transporter nupC, (vc2352) [127]. The *E. coli* homolog of ycdZ, a putative inner membrane Nup protein [128] was also repressed, while the other two putative nupC homologs (vca0179 and vc1953) were not. Gumpenberger and coworkers have recently reported that all three nupC homologs are repressed by CytR in *V. cholerae* [129]. However, these experiments were performed in minimal media using a transcriptional reporter, which may account for the observed differences. The promoters of these five CytR-repressed genes each have the canonical motif for direct CytR anti-activation (Figure 19). The remaining four genes experimentally shown to be directly repressed by CytR in *E. coli* (ppiA, deoC, rpoH and

nupG) either lack an obvious *V. cholerae* homolog (nupG and ppiA), or do not appear to be under CytR control in *V. cholerae* and lack a typical CytR-binding motif (deoC and rpoH). These results are consistent with *V. cholerae* CytR serving as a negative regulator (anti-activator) of nucleoside scavenging by direct binding.

Based on our prior results that CytR positively regulates *comEA* and *pilA* in conditions where TfoX is induced [114], we hypothesized that additional competence genes may be CytR-controlled in a *tfoX** strain, and therefore compared the transcript abundance of different genes in the TfoX* CytR^{+} strain to the isogenic TfoX* CytR^{-} strain. A total of 42 genes showed negative regulation by CytR, including the nucleoside metabolism genes (Figure 7B column 2 blue, and Table 6). However a large number of genes, 84, were also positively regulated (Table 6). In particular, the set of upregulated genes included the transcription factor *qstR*, 15 (of 21) known genes required for natural transformation; 21 genes for the type VI secretion system (T6SS); 4 (of 5) predicted or experimentally validated chitinases; and one chitin utilization gene (Figure 8B column 2 yellow), discussed below.

2.5.4 CytR positively regulates the majority of known transformation genes

Prior studies have identified 21 genes encoded in 11 loci that are necessary for efficient natural transformation [5, 130]. Under conditions in which *tfoX* was induced, CytR upregulated 15 of these 21 genes by $\geq$2 fold (Figure 8B column 2, yellow). Consistent with our prior study, *comEA* (*vc1917*) and *pilABCD* (*vc2423-6*) were positively regulated by CytR; in addition to *pilE* (*vc0857*), *vc0858-vc0861*, and *pilMNOPQ* (*vc2634-30*).

Interestingly, the HapR-controlled regulatory factor gene *qstR* (*vc0396*) was also upregulated 12-fold by CytR (Table 6). These results confirmed that CytR plays a major role in regulating competence genes in *V. cholerae*. In contrast, *pilF* (*vc1612*), *dprA* (*vc0048*), *comEC* (*vc1879*), *comF* (*vc2719*), *pilT* (*vc0462*), and *recA* (*vc0543*) were not under CytR control (Figure 8B, column 3 and 4).

Because the *pilF* homolog, *dprA*, and *comEC* were described as upregulated in response to TfoX induction in El Tor A1552 [5, 130], we also examined our transcriptome data set to determine the effect of *tfoX** induction in the C6706 TfoX* CytR^{+} strain as compared to the isogenic TfoX^{+} CytR^{+} strain carrying the native *tfoX* allele in LB medium. We observed a larger number of genes both positively (108 genes) and negatively (25 genes) regulated by TfoX induction, allowing a comparison of our results with those obtained with El Tor strain A1552 [6, 84]. Each CytR-controlled competence gene we identified (in Figure 8B column 2) was also induced by TfoX, consistent with prior studies [6, 84, 130] (Table 6); along with *pilF* and *dprA*, which were upregulated by TfoX but CytR-independent, as in A1552 (Figure 8B column 4 yellow). In C6706 *comEC* was <2-fold induced under all conditions tested (Figure 8, Table 6), in contrast to modest TfoX induction reported in A1552 [113]. We note that our strains are deleted for *luxO* and "locked" at high cell density, unlike the A1552 strains used in previous reports, which may account for these differences.

By comparing the transcript abundance of the TfoX* CytR^{-} strain to the TfoX^{+} CytR^{-} strain, 101 genes were positively controlled, and 47 genes negatively controlled by TfoX induction when *cytR* was absent (Figure 8B column 3 and Table 6). These results are consistent with our prior study showing that despite *tfoX* induction, a Δ*cytR* strain is

unable to transcribe *comEA* and *pilA* [114]. Expression of *comF*, *pilT*, and *recA* were under neither CytR nor TfoX control. These results demonstrate that CytR is a critical regulator of the majority of known natural competence genes in *V. cholerae*, controlling all but two TfoX-regulated natural transformation genes, *pilF* and *dprA*.

2.5.5 Differential regulation of competence genes by CytR, TfoX and QstR.

RNA-seq analyses revealed that both TfoX and CytR positively control the majority of competence genes, Figure 8), including *qstR*, the transcription factor shown to be directly up-regulated at high cell density by HapR [113]. To confirm and expand upon our RNA-seq observations (Figure 8), we constructed luciferase-based transcriptional fusions to the promoters of the following genes (or operons): *qstR*, *pilM*, *pilE*, *vc0858*, *pilF*, *dprA*, *comF*, *comEC*, and *pilT*. To uncouple native *qstR* expression from HapR, TfoX, and CytR control, a constitutively expressed *qstR** allele was also constructed in a manner analogous to the *tfoX** allele (see Materials and Methods). Expression of each reporter and of the previously published *comEA-lux* and *pilA-lux* reporters [110, 114] was measured in a *V. cholerae* Δ*luxO tfoX** ($TfoX^{*}$ $CytR^{+}$ $HapR^{*}$ $QstR^{+}$) strain and in isogenic strains also carrying deletions in *cytR*, *tfoX*, *hapR*, or *qstR* (Figure 8). We find that in agreement with our RNA-seq analysis, competence gene expression falls into four distinct regulatory classes discussed in detail below

In LB medium, a *V. cholerae* Δ*luxO tfoX** ($TfoX^{*}$ $CytR^{+}$ $HapR^{*}$ $QstR^{+}$) strain expressed *qstR-lux* robustly while isogenic Δ*tfoX*, Δ*cytR*, and Δ*hapR* mutants were all severely impaired in expression (Figure 8A, grey bars) while the corresponding Δ*qstR* strain was not significantly impaired for expression (Figure 8A, white bar). Interestingly,

a constitutive *qstR** allele partially restored (about 100-fold increase) expression of *qstR-lux* in the Δ*tfoX* mutant (Figure 8A, compare bars 2 and 7, $p < 0.01$), suggesting that 1) QstR activates its own transcription and that 2) constitutive expression of QstR also largely bypasses the requirement of TfoX for its activation (Figure 8A). Constitutive QstR expression was not however, able to bypass the requirement for CytR or HapR (Figure 8A, black bars). Thus, expression of QstR is under direct control of HapR, and is controlled by QstR itself, TfoX and CytR, although it remains to be determined whether this is via direct binding.

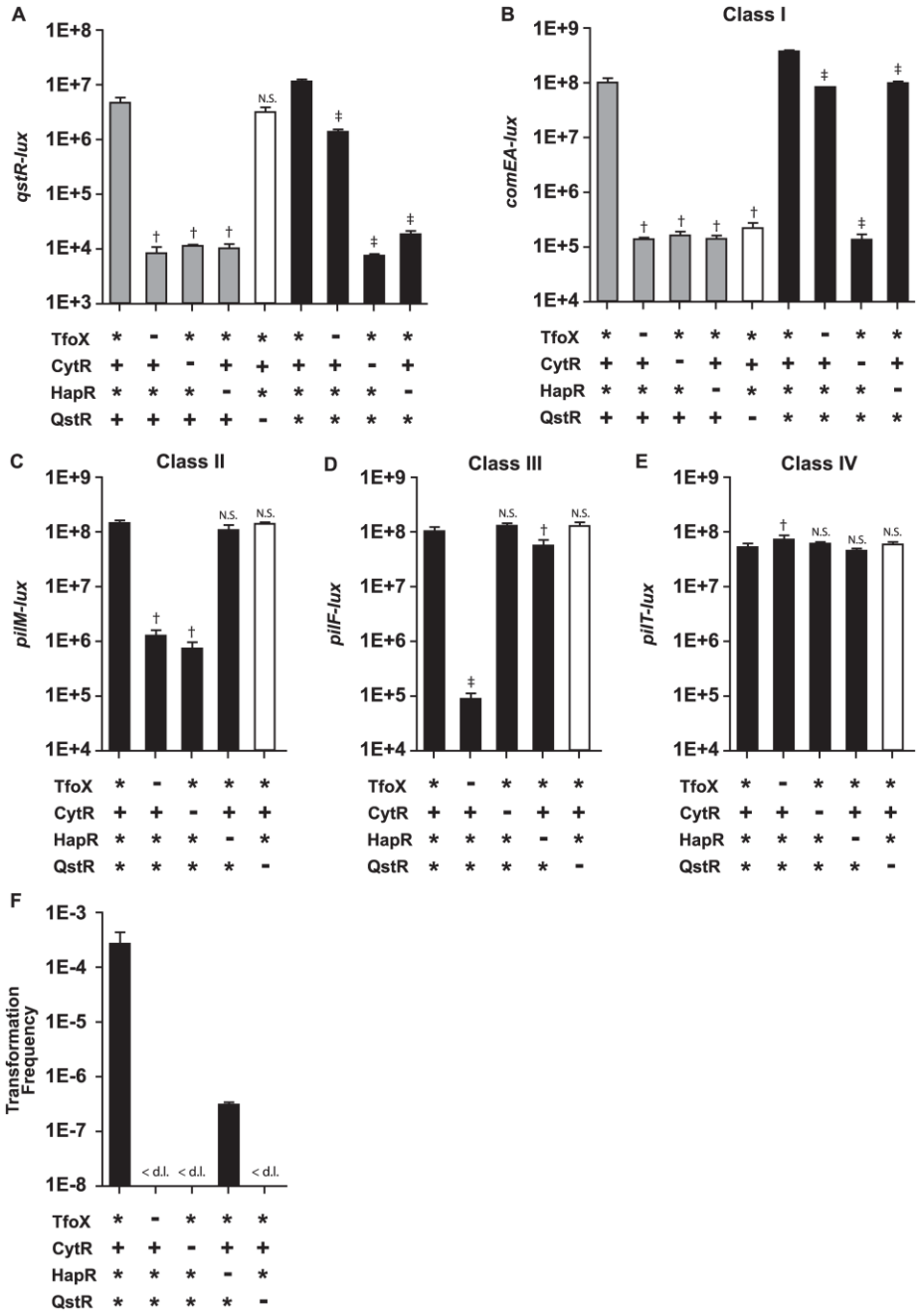


Figure 8: Competence genes are differentially regulated by TfoX, CytR, HapR and QstR.

V. cholerae C6706 derivatives with native alleles of *tfoX*, *cytR* and *qstR* (not constitutively expressed, denoted by +), alleles of *tfoX* or *qstR* made constitutive by replacing the chromosomal native promoter with a *ptac* promoter (indicated by *), or containing in-frame deletions of *tfoX*, *cytR*, *hapR* and *qstR* (-), were analyzed for expression of bioluminescence from plasmid-encoded *lux* transcriptional reporter fusions. Expression profiles are shown for the transcriptional regulator *qstR* (Panel A) and for a member of each regulatory class: class I, *comEA* (Panel B) class II, *pilM* (Panel C) class

III, *pilF*, (Panel D), and class IV, *pilT* (Panel E). All strains are deleted for *luxO* and are **Figure 8 contd:** therefore constitutive for HapR expression (*) when the *hapR* gene is present. Bioluminescence is represented as relative light production per OD_{600} (RLU) and data shown are mean values ± standard deviation from three biological replicates of one representative experiment of three. Data are shown as mean values ± standard deviation. ‡ indicates a p-value < 0.01, † indicates a p-value <0.05. N.S. denotes not significant, calculated using a two-tailed Student's t-test. Panel F: A TfoX* $CytR^+$ HapR* QstR* strain is transformable in LB in the absence of chitin induction, but an isogenic strain carrying a *qstR* deletion was poorly transformable. The *hapR* deletion strain was partially restored for transformation by constitutive expression of QstR (*), but strains deleted for *cytR* or *tfoX* were not restored for competence by the QstR* allele. The limit of detection is 1×10^{-8} cfu. mL^{-1} (d.l.).

We have previously reported that the expression of *comEA* depends upon HapR, TfoX and CytR [114]. However, the requirement of HapR for *comEA-lux* expression was bypassed by constitutive expression of QstR (Figure 8B, compare bars 2 and 7, $p < 0.01$), consistent with our DNA uptake assays (below) and with previous reports [113]. The requirement of TfoX for inducing high levels of *comEA* expression was also bypassed in a *qstR** strain (Figure 8B, compare bars 4 and 9, $p < 0.01$), as also seen by Lo Scrudato and coworkers [109]. By contrast, the requirement of CytR for high levels of *comEA* expression was not restored by constitutive *qstR** expression in a Δ*cytR* strain, indicating that *comEA* is positively regulated by QstR and also independently by CytR. Since individual deletions of *tfoX*, *qstR* and *cytR* all result in loss of *comEA* expression, *comEA* is categorized here as a Class I competence gene, requiring all three regulators for expression. Importantly, the *qstR** allele bypassed a *hapR* deletion for *comEA* expression, but was unable to restore transformation in either a Δ*tfoX* or Δ*cytR* mutant (Figure 8F).

In contrast to *comEA*, which requires all three transcription factors, maximal expression from the promoters of *pilA*, *pilM*, *vc0857* (*pilE*), and *vc0858* required both CytR and TfoX, consistent with RNA-seq results, but not QstR or HapR (Figure 8C, and Table 6) (defined here as Class II competence genes). The requirement of TfoX for expression

from these promoters was not bypassed in the strain carrying the *qstR** allele (Figure 8C) as it was for *comEA* (Figure 8B). We previously reported that *pilA* expression was dependent on quorum sensing control through HapR [114]. Subsequent independent analyses here show that expression of *pilA* (and *chiA1*, discussed below) does not depend on HapR control, consistent with results reported in *V. cholerae* strain A1552 [130] (Figure 20).

RNA-seq results revealed that expression of *pilF* and *dprA* depend on TfoX as shown by others [6, 131], and similar results were obtained with the *pilF-lux* and *dprA-lux* fusions. These two genes did not require CytR, HapR or QstR for maximal expression (Figure 8D, and Figure 20) and were assigned as Class III competence genes. Consistent with RNA-seq, *pilT-lux* was expressed but not altered in strains deleted for each regulator tested, and the *comEC-lux*, and *comF-lux* reporters were not expressed (Figure 8E, and Figure 20); and were deemed Class IV competence genes because they were not under control of any of the four known regulators based on both RNA-seq and *lux*-based reporter assays.

These data suggest distinct roles for both CytR and TfoX in competence regulation, summarized in the Discussion. TfoX up-regulates *comEA* in a QstR-controlled manner thus dependent on quorum sensing (HapR), while CytR upregulates *comEA* via both QstR-dependent and QstR-independent means. 14 competence genes (*pilE, vc0858-0861*, *pilABCD*, and *pilMNOPQ*) are likely regulated by CytR and TfoX in a QstR-independent manner. Also, TfoX up regulates *pilF* and *dprA* in a CytR, QstR and HapR-independent manner.

Consistent with this scheme of regulation, we observed that a (TfoX* CytR$^+$ HapR* QstR*) strain was highly transformable in LB medium, while no transformants were detected for the corresponding Δ*tfoX*, Δ*cytR*, and Δ*qstR* mutants (Figure 8F). A *hapR*- strain was partially complemented for transformation by QstR* induction similar to results obtained for *V. cholerae* strain A1552 [113].

2.5.6 CytR is required for bacterial killing via the Type VI secretion system.

TfoX and QstR positively regulate thirty genes that are encoded in three Type Six Secretion System (T6SS) loci of *V. cholerae* (*vc1415-1421*, *vca0017-0021*, and *vca0107-0124*). The T6SS promotes killing of "prey" cells, and the DNA released by the lysed prey can be used for natural transformation [84]. The inner tube of the T6SS apparatus, which also acts as a chaperone for T6SS effectors, requires the membrane ATPase VasK for secretion [132]. RNA-seq analyses revealed that CytR positively regulates all three T6SS operons (Figure 8B, column 2). To characterize the contribution of CytR and the other regulators (TfoX, HapR, and QstR) to T6SS regulation, we constructed luciferase-based transcriptional fusions to the first promoter of the three T6SS loci: *vc1415*, *vca0017*, and *vca0107*. In contrast to competence regulation, the contribution of TfoX and CytR to upregulation of the type VI secretion system occurs primarily through a QstR-dependent mechanism, discussed below.

Each T6SS reporter was introduced into the Δ*luxO tfoX** strain (TfoX* CytR$^+$ HapR* QstR$^+$) carrying the native *qstR* allele, an isogenic *qstR** strain that expresses QstR constitutively, and derivatives singly deleted for *tfoX*, *cytR*, *hapR*, and *qstR*. Robust expression from the *vca0017-lux* T6SS promoter fusion was observed in the TfoX* CytR$^+$

HapR* QstR+ strain that expresses QstR from its native promoter (Figure 9A). Expression levels were modestly reduced in the corresponding TfoX- and CytR- strains, supporting the interpretation that CytR, like TfoX, plays a significant role in T6SS gene expression [84]. Expression was lowest in the isogenic HapR- strain, consistent with reports of at least three distinct levels of quorum sensing dependent regulation of T6SS genes: direct binding by quorum regulatory small RNAs [97] and HapR [133], and through QstR [84]. The QstR- strain unable to auto-activate was reduced relative to the parental QstR+ strain. Surprisingly, expression of the constitutive *qstR** allele bypassed deletion of each of the other four regulators (Figure 9A, black bars). A similar pattern of expression with minor differences was also obtained for luciferase fusions to the other two T6SS promoters (Figure 21). Thus, heterologous *qstR* expression, uncoupled from its native regulatory role, is sufficient for T6SS gene expression.

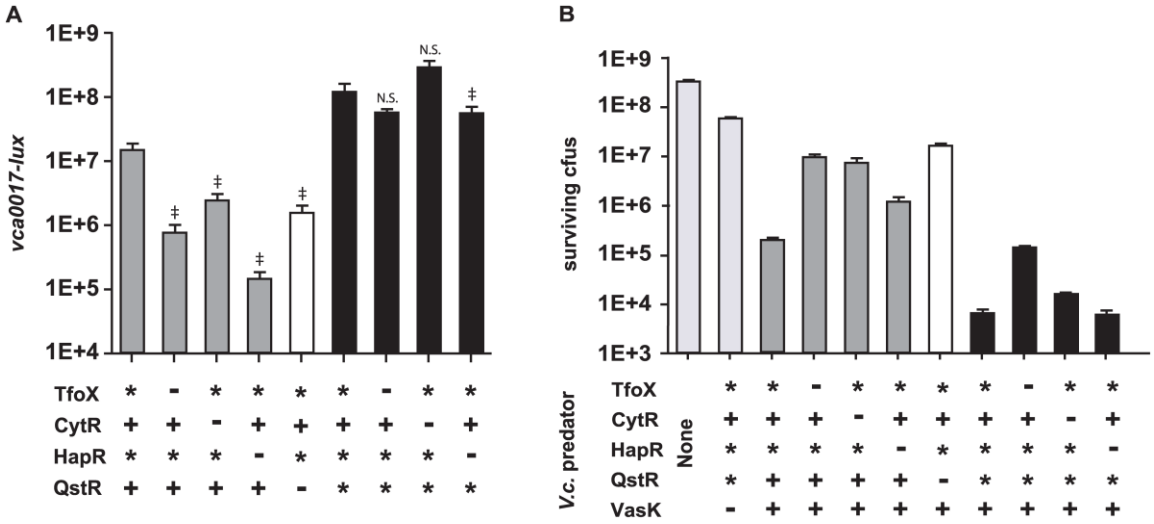


Figure 9: Expression of Type VI secretion system genes and T6SS-mediated killing are positively regulated by CytR, TfoX, HapR, and QstR.

V. cholerae C6706 with indicated alleles of *tfoX*, *cytR*, *hapR*, and *qstR* (+, native; -, deletion; *, constitutively expressed) were analyzed for expression of bioluminescence from a plasmid-encoded *lux* transcriptional reporter fusion to the promoter of first gene of

a T6SS auxiliary cluster, *vca0017* (Panel A). Bioluminescence is defined as relative light production per OD_{600} (RLU). All strains are deleted for *luxO* and are therefore constitutive for HapR expression (*) when the *hapR* gene is present. Data shown are mean values ± standard deviation for triplicates from one representative experiment of three performed. ‡ indicates a p-value < 0.01, † indicates a p-value <0.05. N.S. denotes not significant, , calculated using a two-tailed Student's t-test. Panel B: Chloramphenicol resistant *E. coli* prey were incubated with the indicated *V. cholerae* predator strains at a ratio of 1:10 on membrane filters to monitor contact-dependent killing. Total surviving prey cfus are represented in each case.

Next we investigated whether the type VI secretion system was functional when induced for *tfoX*, *qstR* and *hapR* expression in C6706. Previous reports for the C6706 strain demonstrated that WT C6706 does not express T6SS in LB and is unable to kill *E. coli* [84, 86]. Interspecies killing assays between *V. cholerae* predator and *E. coli* prey performed as described previously [56] revealed robust killing of *E. coli* prey by both the $TfoX^{*}$ $CytR^{+}$ $HapR^{*}$ $QstR^{+}$ and $TfoX^{*}$ $CytR^{+}$ $HapR^{*}$ $QstR^{*}$ strains (Figure 9B), but not a *vasK* deletion mutant that is unable to effectively secrete the Hcp subunit of the inner tube of the T6SS [132] or C6706 uninduced for TfoX and HapR (data not shown). Killing was severely impaired in the isogenic $TfoX^{-}$ and $CytR^{-}$ strains consistent with the role of both regulators in T6SS-dependent killing, and the HapR- showed a modest killing defect. By contrast the $QstR^{-}$ strain exhibited no killing, similar to that of the $VasK^{-}$ strain. Consistent with the transcriptional reporter results observed with the *qstR** strains (Figure 9A), constitutive *qstR** expression bypassed deletions of each of the other regulators for T6SS-mediated killing (Figure 9B, black bars). This mechanism of regulation is distinct from that of transformation. Notably, for T6SS, signal transduction from growth on chitin (via TfoX) and from nucleoside starvation (via CytR) are mediated primarily through QstR; in contrast to TfoX- and CytR-control of transformation, which

occurs both through QstR-dependent and QstR-independent pathways (see discussion for details).

2.5.7 CytR and TfoX co-regulate expression of chitinase genes.

V. cholerae has five genes encoding predicted chitinases that may participate in degradation of chitinous material, such as crab and shrimp shells and zooplankton molts [105, 134]. We showed previously that CytR positively regulates expression of a *chiA1-lux* reporter. TfoX was also identified as a critical activator of several predicted or validated chitinase genes (*chiA-1*, *chiA-2*, *vc0769*, and *vca0700*), but not the fifth predicted chitinase *vc1073* [6, 105]. Consistent with these findings, robust up-regulation of each of these four chitinases (but not *vc1073*) was observed in the $CytR^{+}$ $TfoX^{*}$ strain, compared to corresponding strains lacking CytR and TfoX (Figure 8B). To investigate the effect of CytR and TfoX on expression of chitinase genes, we constructed transcriptional luciferase fusions to the promoters of each chitinase and measured expression of these reporters (and of the previously constructed *chiA1-lux* reporter) in a Δ*luxO tfoX* qstR** ($TfoX^{*}$ $CytR^{+}$ $HapR^{*}$ $QstR^{*}$) strain and in isogenic strains carrying deletions in *cytR*, *tfoX*, *hapR* or *qstR*. Maximal expression of each of the four chitinases occurred in the $TfoX^{*}$ $CytR^{+}$ $HapR^{*}$ $QstR^{*}$ strain. Deletions in *cytR* or *tfoX* greatly impaired expression of each reporter, but deletion of *qstR* or *hapR* did not significantly impact chitinase expression (Figure 10A, and Figure 22), suggesting that chitinase expression, like Class II competence gene expression, is controlled by TfoX and CytR, but not HapR or QstR.

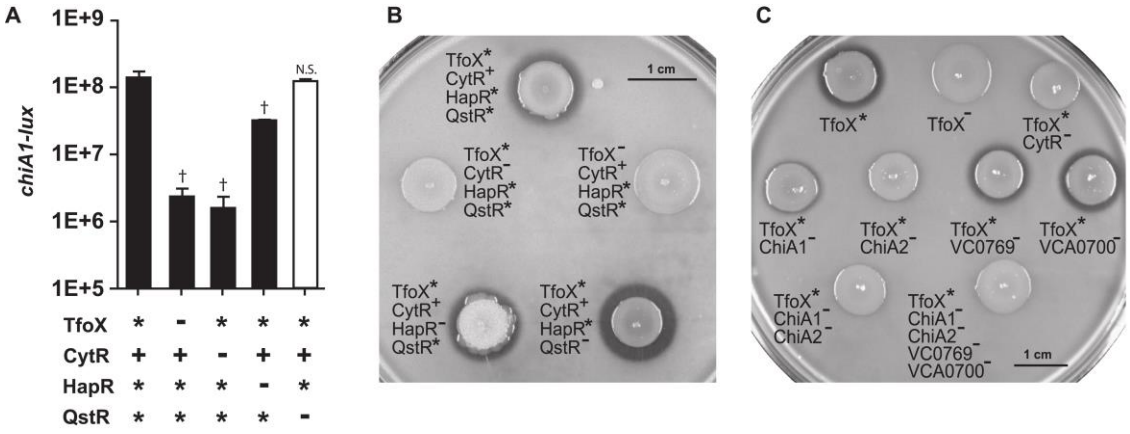


Figure 10: Expression of *V. cholerae* chitinases requires TfoX and CytR, but not HapR or QstR.

Panel A: *V. cholerae* strains with indicated alleles of *tfoX*, *cytR*, *hapR* and *qstR* (+, native; -, deletion; *, constitutively expressed), were analyzed for expression of bioluminescence from a plasmid-encoded *lux* transcriptional reporter fusion to the promoter of the chitinase *chiA1*. All strains are deleted for *luxO* and are therefore constitutive for HapR expression (*) when the *hapR* gene is present. Bioluminescence is defined as relative light production per OD_{600} (RLU). ‡ indicates a p-value < 0.01, †indicates a p-value <0.05. N.S. denotes not significant, calculated using a two-tailed Student's t-test. Panel B and C: Chitin agar plate assays. *V. cholerae* strains with indicated alleles of *tfoX*, *cytR*, *hapR*, and *qstR* were assayed for chitinase activity which results in a zone of clearing on LB plates containing 2% colloidal chitin (panel B). Strains constitutive for TfoX (*) and isogenic strains deleted for *cytR*, *tfoX* and the CytR-dependent chitinases *chiA1*, *chiA2*, *vc0769*, *vca0700*, a *chiA1 chiA2* double mutant and a strain deleted for all four chitinases were assayed for the contribution of individual chitinase genes to chitinase activity (panel C).

To determine the effect of CytR on the ability of *V. cholerae* to utilize chitin, we performed a chitin agar plate assay [125]. A $TfoX^{*}$ $CytR^{+}$ $HapR^{*}$ $QstR^{*}$ colony was able to produce a zone of clearing by degradation of colloidal chitin (Figure 10B), while the isogenic $TfoX^{-}$ and $CytR^{-}$ strains did not, indicating that the presence of both CytR and TfoX is necessary for metabolizing chitin. Isogenic $HapR^{-}$ and $QstR^{-}$ strains were able to clear chitin, confirming that quorum sensing is not required for chitinase activity (Figure 10B,Figure 22). We also tested $TfoX^{*}$ strains singly deleted for each CytR-controlled

chitinase (Figure 7B), for the ability to degrade chitin. A TfoX* ChiA-1^{-} mutant produced a decreased zone of clearing while TfoX* ChiA-2^{-} produced a very slight zone of clearing (Figure 10C and Figure 22). In contrast TfoX* VC0769^{-} and TfoX* VCA0700^{-} strains were not impaired for chitin degradation. Consistent with their predicted role as the dominant extracellular chitinases [105], a ChiA-1^{-}, ChiA-2^{-} double mutant produced no zone of clearing, identical to a strain deleted for all four chitinases. (Figure 10C, Figure 22). Thus, although CytR upregulates four chitinase genes, *chiA1* and *chiA2* appear to be sufficient for degrading colloidal chitin.

2.6 Discussion

We have demonstrated that in *V. cholerae*, the regulator CytR not only represses genes involved in nucleoside metabolism and transport, but also positively regulates natural transformation, the type VI secretion system, and chitin degradation indicating novel roles for CytR regulation. Although each of these phenotypes requires CytR for expression, the specific mechanism of regulation and the involvement of other transcription factors appear to differ in each case (Figure 11).

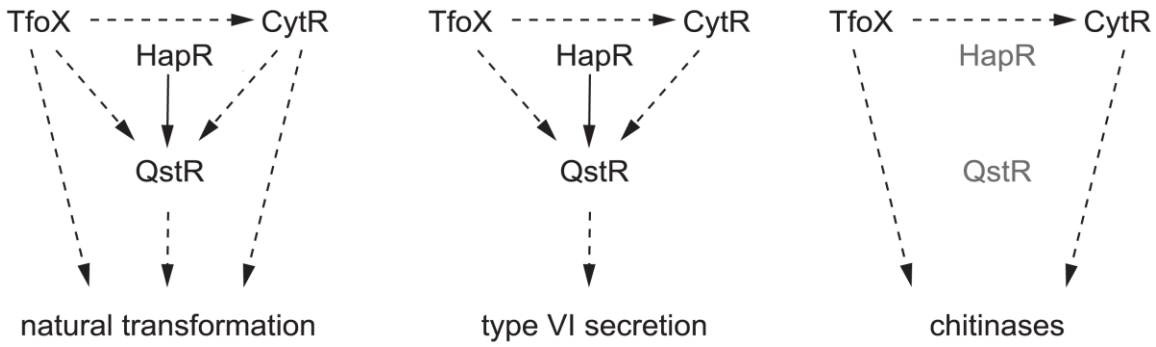


Figure 11: The differential roles of TfoX, CytR, HapR and QstR in natural transformation, Type VI secretion and chitinase expression.

Natural transformation requires inputs from four regulators TfoX, CytR, HapR and QstR. Type VI secretion requires inputs from the four regulators above, but QstR overexpression bypasses the need for TfoX, CytR, and HapR. Chitinase expression requires inputs from TfoX, CytR but not from HapR and QstR under the conditions tested.

Natural transformation requires CytR, chitin-induced TfoX, and quorum sensing-mediated QstR (Figure 11). The majority of competence genes (Class II) require TfoX and CytR for expression but not QstR, while a smaller subset of competence genes are maximally expressed in the presence of all three regulators (Class I) or only TfoX (Class III). The requirement of high cell density conditions (quorum sensing) for regulation of *comEA*, but not for expression of the transformation pilus, suggests a separation in the events of transformation. While CytR and TfoX suffice for the assembly of the transforming pilus and for DNA uptake into the periplasm, subsequent entry into the cytoplasm and potential acquisition of genetic material is contingent upon growth at high cell density. The extracellular DNAse Dns is produced at low cell density, reduces transformation efficiency [112], and is important for utilization of DNA as a phosphate source [135], suggesting that at low cell density, extracellular DNA may be more useful

to *V. cholerae* in nutrient supply rather than as a source for acquisition of genetic material by horizontal transfer.

In contrast to competence genes, expression of the three T6SS clusters requires TfoX, CytR, HapR and QstR for maximal expression (Figure 9). Quorum sensing control of T6SS expression appears to act multiple levels, via *qrr*s, HapR and QstR [84, 97, 133]. CytR and TfoX are necessary for T6SS expression but their regulatory effects are mediated solely via QstR-upregulation (Figure 11). This suggests a central role for quorum sensing control of killing mediated through T6SS, consistent with a function for T6SS in inter- or intra-species antagonism or in acquiring DNA for natural transformation by lysis of related neighbors [84, 136]

We observed that expression of the four described chitinases in *V. cholerae* is dependent on TfoX and CytR and is independent of HapR- and QstR-mediated quorum sensing control, similar to the Class II competence genes (Figure 11). Sun and coworkers observed modest HapR-mediated transcriptional repression of two chitinases, *vca0027* (*chiA-2*) and *vc0769* [137]. However, their experiments were performed with *V. cholerae* biofilms on chitin flakes and other levels of signaling may operate under these conditions. These results are consistent with a requirement for chitinase activity both at low cell density during biofilm establishment on chitinous surfaces and at high cell density to ensure a continuous source of carbon.

While expression of TfoX is up-regulated by growth on chitin and QstR expression depends upon both chitin and growth at high cell density, the environmental signals that modulate CytR-dependent regulation are uncertain. TfoX induction increases

expression of *cytR* ~2-fold (Table 6 and [6]), thus it remains possible that growth on chitin may serve as an activating signal for *cytR* transcription in *V. cholerae*. Post-transcriptional regulation results from allosteric binding of cytidine to CytR, which causes de-repression of CytR-repressed targets. In *E. coli*, the in vitro binding affinity of CytR for its allosteric ligand cytidine is ~2 μM [138], however de-repression of CytR-repressed targets *in vivo* in both *E. coli* and *V. cholerae* requires cytidine supplementation at millimolar concentrations unlikely to occur in the environment [114, 139]. It remains possible that the relevant environmental signal may be a modified pyrimidine nucleoside that is more efficiently imported (or less efficiently degraded) and acts as a stable CytR-ligand. Alternatively, CytR may regulate its targets in response to fluctuations in the intracellular nucleotide pools which are known to vary with growth rate and during the stringent response [140].

The mechanism by which CytR up-regulates the phenotypes described here remains to be determined. Rasmussen and coworkers have demonstrated that CytR has the potential to act as a modest transcriptional activator by stabilizing CRP at an artificial weak CRP-binding site [34], but no naturally occurring examples of this type of promoter have been reported. Instead, *in vitro* studies have shown that CytR from *E. coli* acts almost exclusively as a repressor (or anti-activator) at a subset of CRP-activated promoters [115]. As a result, we initially proposed that CytR may indirectly act as an activator by repressing another transcriptional factor that directly represses competence genes [114]. However, we have not been able to identify a candidate repressor by genetic screens or chromatin immunoprecipitation followed by high throughput sequencing (chIP-seq) (data not shown). Several genes are differentially regulated at the

transcriptional and post-transcriptional level by the relative abundance of their initiating nucleotide [141-143]. Thus it remains possible that CytR may maintain high intracellular UTP and CTP concentrations by repressing pyrimidine catabolism, thereby promoting transcription of genes with pyrimidine initiation nucleotides.

In conclusion, we have demonstrated that CytR, previously thought to function almost exclusively in the nucleoside scavenging response, also regulates genes under chitin and quorum sensing control. Further studies are needed to determine both the relationship between these environmental signals and the molecular mechanism by which this regulation occurs. CytR in *V. cholerae* controls multiple behaviors that are important for its fitness and adaptability in the environment.

CHAPTER 3. Computational characterization of type VI secretion systems in *Vibrio cholerae*

3.1 Abstract

The human pathogen *Vibrio cholerae* employs several adaptive mechanisms for environmental persistence including natural transformation and type six secretion, creating a reservoir for the spread of disease. Here we report whole genome sequences of 23 diverse *V. cholerae* isolates, effectively doubling the number of publicly available environmental *V. cholerae* genomes.

3.2 Introduction

Vibrio cholerae is a globally dispersed, Gram-negative bacterium found in water and the stool of infected animals and the causative agent of diarrheal disease cholera in humans. While relatively rare in developed countries[144, 145], cholera is endemic to areas of the Middle East and Southern Asia causing tens of thousands of cases each year [146, 147]. Clinical isolates of *Vibrio cholerae* possess several virulence factors including the phage-derived cholera toxin (CTX) and toxin co-regulated pilus (TCP) that help colonize the intestine and infect the human host [148]. In addition to these virulence factors, *V. cholerae* also possesses several mechanisms to colonize and persist in its aquatic niche. Chief amongst these is the presence of the Type VI secretion system (T6SS), which is a protein delivery system that can translocate protein cargo across membranes of neighboring cells [53]. The T6SS has been variously described as a needle [149] or crossbow [150], and the delivered protein effectors can induce toxicity or lysis in neighboring prokaryotic or eukaryotic cells (Figure 6) [53, 54]. Most of the structural

components of *V. cholerae* T6SS are encoded in a single operon on chromosome II, called the large cluster. Additional components such as two Hcp proteins that make up the internal sheath are encoded on auxiliary clusters – Auxiliary clusters 1 and 2 (Aux 1, 2) respectively. Each of the three clusters encodes a valine-glycine repeat containing protein VgrG, which adorns the tip of the type six apparatus and is critical for assembly of a functional system (Figure 6)[61]. Some VgrG proteins may contain additional domains that possess antibacterial (lysozyme-like) or anti-eukaryotic (actin-crosslinking domain) properties [62]. Additionally, in all *V. cholerae* genomes investigated to date, the penultimate coding sequence (CDS) of each cluster encodes a toxic effector protein which is delivered directly into the periplasm or cytoplasm of neighboring cells. This is followed by a cognate immunity protein which protects against self-intoxication or killing by sister cell and loss of cognate immunity proteins makes cells susceptible to T6SS attacks from neighboring cells [60, 62]. Each auxiliary cluster also encodes a DUF4123-containing adaptor protein directly upstream of the effector that is thought to be critical in loading the effectors onto the T6SS apparatus [65, 66]. Due to this conserved genomic organization (synteny), adaptor proteins have been used as genomic markers to identify novel T6SS effectors [65, 66]. An additional cluster, Aux 3, identified by secretome analysis [151] displays a non-canonical organization that lacks hcp, vgrG, and tap genes. Instead of a TAP protein, each Aux 3 cluster encodes gene for a PAAR-repeat protein that forms the tip of the T6SS assembly, followed by the gene for a putative hydrolase effector, tseH, which is toxic to *E. coli* cells, and then a cognate immunity gene, tsiH. Unterweger et al. [62] studied the T6SS loci from 37 sequenced *V. cholerae* strains (25 clinical and 12 environmental isolates) and reported diversity in

"effector modules", which contain effector-immunity proteins. The majority of toxicogenic clinical isolates encode the same set of effector/immunity pairs and are compatible due to conservation of similar immunity factors. In contrast, non-toxicogenic environmental isolates are highly diverse in the effector content and shown to be incompatible when grown in co-culture due to dissimilar immunity factors. Similarly, in a recent survey Bernardy et al [4] found that a majority of environmental isolates sampled from diverse locales killed *E. coli* prey constitutively with varying efficacy, while only a minority of clinical isolates could do so. It has been proposed that the distinct regulatory differences between clinical and environmental *V. cholerae* may reflect adaptation of some strains to the human intestine where T6 activity may be highly regulated and other strains to the aquatic niche outside the human host where constitutive T6 activity may be advantageous [4, 82, 83, 152]. To better understand the genetics that underlie these diverse *V. cholerae* T6 phenotypes detailed in Bernardy et al, we sequenced 23 environmental and 3 clinical isolates. Here we present 1) a new tool for rapid identification and annotation of T6SS in *V. cholerae,* 2) a network-based approach for identification and classification of T6SS effectors and 3) the discovery of two previously unreported T6S loci.

3.3 Materials and methods

Strain and assembly information are summarized in Table 3 . All strains were grown overnight in LB Medium (Difco) at 37 °C, with shaking. Genomic DNA was isolated using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research) and paired-end fragment libraries constructed using Nextera XT DNA Library Preparation Kit (Illumina) with a fragment length of 300bp. Libraries were sequenced by the High Throughput Sequencing

Core at Georgia Institute of Technology on an Illumina HiSeq 2500 Rapid platform, producing approximately 280 million 100bp reads in total. Reads were trimmed using Trimmomatic [153] to remove adapters and bases with read quality score <20. Genomes were assembled using SPAdes v3.5 [154] and annotated using the Rapid Annotation and Subsystem Technology (RAST) web tool provided by National Microbial Pathogen Data Resource [155-158]. T6SS genes were annotated using T6SS Predictor [159, 160].

3.3.1 Nucleotide sequence accession numbers

This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession numbers listed in Table 3. 14 publicly available, completed *Vibrio cholerae* genome sequences were downloaded from National Center for Biotechnology Information's (NCBI) RefSeq sequence collection[161] and 31 additional incomplete genomes and sequence reads archives were retrieved from NCBI's GenBank [162] collection and Pathosystems Resource Integration Center (PATRIC)[157]. RefSeq accessions: GCA_000006745.1, GCA_000016245.1, GCA_000021605.1, GCA_000022585.1, GCA_000166455.2, GCA_000195065.1, GCA_000250855.1, GCA_000765415.1, GCA_000829215.1, GCA_000963555.1, GCA_000969235.1, GCA_000969265.1, GCA_001045415.1. GenBank accessions: ACFQ00000000, AAUR01000000, AAUT01000000, AAWG00000000, AATY01000000, AFSV00000000, ACIA00000000, AHGQ00000000, KF228943/KF228947/KF228950, ACVW00000000, SRA037374, KC955251/KF228941/KF228945, KF228942/KF228946/KF228949, SRA035959, SRA035998, SRA035995, SRA030739, ALDP01000000, ALEC00000000, SRA030720, ALED00000000, AAUS00000000, AAKF03000000, AAWF01000000, AAUU01000000, KF228944/KF228948/KF228951,

ACHW00000000, ACHY00000000, AAKI02000000, AAKJ02000000, and ACHV0000000.

Table 3: List of strains sequenced in this study.

The strain name, location, source and year of isolation, presence/absence of type VI killing activity and the NCBI accession numbers are shown. Majority of the strains were isolated from environmental source except strain C6706 and V52.

Strain	Location	Source	Year of isolation	Type VI killing activity[4]	NCBI Accession
1496-86	USA (LA)	Moore swab	1986	-	MIPC00000000
2523-87	USA (LA)	Moore swab	1974	-	MIPE00000000
VC48	USA (FL)	Oyster	1981	-	MIOT00000000
MZO-02	Bangladesh	Patient	2001	-	MIKJ00000000
2633-78	Brazil	Sewage	1978	-	MIPH00000000
3272-78	USA (MD)	Water	1977	-	MIOZ00000000
TP	USA (CA)	Water	2000	-	MIPK00000000
3568-07	Mexico	Queso fresco	2007	+	MIPL00000000
2559-78	USA (LA)	Crab	1978	+	MIOW00000000
HE46	Haiti (Centre)	Gray water	2011	+	MIPM00000000
2479-86	USA (LA)	Moore swab	1986	+	MIPB00000000
2497-86	USA (LA)	Moore swab	1987	+	MIPD00000000
2512-86	USA (LA)	Moore swab	1986	+	MIOY00000000
2631-78	USA (LA)	Moore swab	1978	+	MIOX00000000
VC22	USA (FL)	Oyster	1981	+	MIKK00000000
VC53	USA (AL)	Oyster	2009	+	MIOU00000000
VC56	USA (AL)	Oyster	2009	+	MIOV00000000
1074-78	Brazil	Sewage	1978	+	MIPG00000000
3223-74	Guam	Storm drain	1974	+	MIZG00000000
3225-74	Guam	Storm drain	1974	+	MIPF00000000
2740-80	USA (Gulf coast)	Water	1980	+	MIKI00000000
692-79	USA (LA)	Water	1979	+	MIPA00000000
SIO	USA (CA)	Water	2000	+	MIPJ00000000
C6706	Peru	Patient	1991	-	MIPI00000000
V52	Sudan	Patient	1968	+	MIPN00000000

3.3.2 Whole genome comparisons

The 14 RefSeq genomes and 26 genomes from this study were subjected to an all-by-all nucleotide comparison using one-way, reciprocal best hits BLAST [163] to calculate percent identity between 1024bp blocks generated from each genome sequence. The average nucleotide identity by BLAST (ANIb) [164, 165] was computed for each one-way, pairwise comparison and the lower ANI value for each given pair was retained. A 30x30 symmetric matrix of ANIb values was constructed and hierarchically clustered by complete linkage and heatmap generated in R [166] using the ggplot2 [167] package. To assess genome rearrangements or duplications, dot plots were generated for some genomes using the MUMmer package [168, 169] (Figure 23)

3.3.3 Computational characterization of T6SS

Initial identification and annotation of Large and Auxiliary T6SS clusters was by BLAST against a database constructed using sequences reported previously by Unterweger et al. [62] and Altindis et al. [151]. Briefly, VrgG, hcp, and effector alleles were retrieved from 37 previously studied *V. cholerae* strains, deduplicated, and used to construct protein BLAST databases. Predicted genes from RAST were searched against the constructed databases using BLASTp and tBLASTx [163]. VrgG-3 alleles belong to the Large cluster, while VrgG-1 and 2 alleles, which are practically indistinguishable at sequence level, served as markers for putative Aux 1 or 2. BLAST hits to effector proteins were considered true positives if within +/- 3 CDS of a predicted VgrG and in the same orientation. When genes for VgrG proteins were identified with no neighbouring effector annotation, genes at the +/-2 and +/-3 CDS were labelled putative effector and immunity proteins, respectively, and marked for manual validation. All initially identified loci were

manually validated, with new VgrG and Effector alleles incorporated into the BLAST database. Manual validated involved cross-checking domain and BLAST hit with expected combinations. A T6 SS cluster was not defined by the presence of an *hcp* gene presence, but was by contiguous genes for a VgrG, TAP, effector and immunity protein. This iterative method was repeated until no new, validated clusters were found.

Putative effector functional annotations were assigned on the basis of conserved functional domains. Reverse, position specific BLAST (rpsBLAST) against the Protein Families (Pfam [170]), Cluster of Orthologous Groups (COG, [170]), and Conserved Domain Database (CDD, [171, 172]) databases was used to identify characteristic domains. Effectors were assigned to the following classes: VgrG-3, lipase, hydrolase (VasX), NTPase/transferase, LysM, and TseH (Figure 13 and Figure 14)

To identify additional T6SS clusters, the refined databases of validated effector and VgrG alleles were transformed into Hidden Markov Models (HMM). The HMMer suite [173][172][172][172][172][172][172][172] [172][164] provides a convenient, command line interface for the creation and use of HMMs with genomic data. Profile HMMs were constructed for each of the effector classes above using the 37 strains from Unterweger et al. and the 26 strains sequenced strains. Additionally, two profile HMMs were produced for each *hcp* and *vgrG*1/2 alleles and designated as hcp_degenerate, and vgrg_degenerate, respectively. The *hcp* and *vgrG* HMMs were built as before using the refined BLAST databases. Hcp_degenerate was built using functionally related sequences from other microbial and plant pathogens obtained by psiBLAST against the NR/NT database [174] and validated by literature implicating them in T6S. Vgrg_degenerate was built using computationally produced chimeras, truncations and mutations of VgrG sequences based

on VgrG-1/2 sequences from this study. As before, hits were validated by presence of a *vgrG* and putative effector gene within a few CDS of each other and Pfam/domain content. The annotation workflow can be found in Figure 24. Auxiliary clusters 4 and 5 were additionally validated by PCR and Sanger sequencing to confirm sequence content.

3.3.4 T6SS effector networks

Protein networks were derived from Smith-Waterman local alignment data. Effector alleles from Aux 1, 2, and 3 and the two additional clusters we have designated Aux 4 and 5, were subjected to all-by-all pairwise Smith-Waterman local alignment [175] using 'water' from the EMBOSS suite [176]. Each pairwise comparison was assigned an edge weight equal to the pairwise distance between nodes, (1- fraction identity), in the initial network. To determine the edge-weight cutoff to use, all-by-all pairwise comparisons were conducted for every annotated gene in a given genome with at least one matching annotation in another genome and a density plot of their identities constructed (Figure 25). Nearly all genes with shared annotation were >95% identical. Edges with weights > 0.8, corresponding to identity < 0.2, were discarded because, in general, sequence identity < 20% indicates evolutionarily unrelated sequences [177]. The networks were imported into Cytoscape [178] and visualized using the Force Directed layout.

3.4 Main findings

T6SS loci were annotated in all genomes in an effort to characterize the genetic basis of T6SS-mediated killing among diverse environmental *V. cholerae* isolates. All genomes were found to encode the previously characterized large cluster and two auxiliary clusters, which together comprise the canonical T6SS loci. In addition, two previously unreported T6SS loci were discovered in six of the isolates. Numerous examples of novel effector-immunity protein pairs, which function together to catalyze T6SS-mediated killing, were characterized among the set of environmental isolate genomes. Taken together, our genome analysis illuminates the diverse repertoire of genetic mechanisms that underlie T6SS-mediated killing in *V. cholerae*.

3.4.1 Whole genome comparison and diversity assessment

Average Nucleotide Identity (ANI) has replaced DNA-DNA Hybridization as the species typing tool in the genomic era [165]. BLAST based ANI (ANIb) has a relatively strict species cutoff, with ANIb values < 0.96 indicating different species [164, 165, 179]. ANIb revealed 6 clusters of *V. cholerae* strains (Figure 12). SIO (BH2680), the out group, had an ANI value close to 0.96 and is at the edge of the *V. cholerae* species boundary. As expected, clinical isolates of *V. cholerae* clustered together, likely due to their clonal nature [146, 180-182]. ANIb values >99% are often used for subspecies or strain delineation [183], further supporting the clonal origins of clinical samples. The clusters revealed by ANI roughly correlate with the types of T6SS effectors each strain possesses (Figure 12).

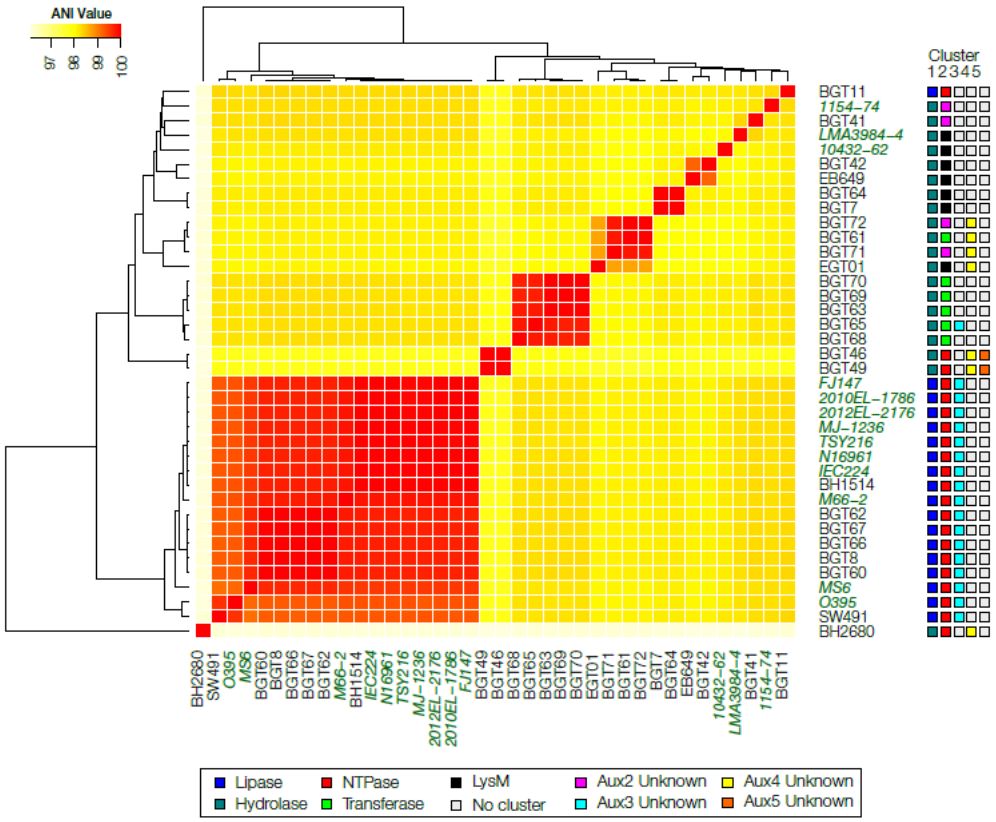


Figure 12. Six clusters of similar strains are revealed by all-against-all Average Nucleotide Identity.

The matrix (a) includes the 26 strains from this study and 14 high quality publicly available reference *V. cholerae* genomes from NCBI (depicted in green). ANI uses one-way and reciprocal best hits to determine protein identity between sequences. Strains which cluster together share similar phenotypes and Type VI Secretion effector-immunity proteins. A phylogenetic tree (b) constructed from the ANI.

Dot plots between strains in different ANI clusters show few, small (<20kb) rearrangements and many, small unique genomic regions (data not shown); consistent with previously observed high rates of horizontal gene transfer that may be due to natural competence in *V. cholerae* [184-186]. Dot plots of strains within the fourth ANI group (BGT61, 71, 72, EGT01) showed no large inversions or insertion/deletions and high

conservation of sequence (Figure 23). Strains BGT61, 71 and 72 were collected in the same year, 1978, from sites approximately 5000 mi apart (Table 1). BGT71 and 72 were collected from sewage runoff while BGT61 was cultured from crab caught off the coast of California. This suggests environmental reservoirs contain well-mixed populations of *V. cholerae* that may be distributed broadly by ocean currents. Interestingly, EGT01 was collected 33 years later from grey water (water from non-sewage, home water sources; e.g. kitchen sink) in Haiti following the 2010 cholera outbreak and shares many of the same genomic features. However, EGT01 also encodes two bacterial CRISPR systems, including one upstream of a T6SS cluster.

3.4.2 T6SS module typing and annotation

V. cholerae T6SS loci have highly conserved structure and gene order. The last four genes of the large cluster and the entire Aux 1, 2 and 3 loci are depicted in Figure 13. The conserved gene order was used to localize BLAST searches around BLAST-annotated VgrG proteins to reduce the required number of searches. Initial BLAST annotation against previously reported effector sequences [62, 151] was partially successful. VgrG alleles of the Large, Aux 1 and 2 clusters were successfully annotated in the majority of strains, with occasional mis-annotation of VgrG-1 proteins as VgrG-2 and vice versa. However, some Aux 1 effectors belonging to the hydrolase class and Aux 2 effectors belonging to the LysM class were left unannotated. Aux 3 effector/immunity proteins, previously reported by Altindis, et al.[151, 152], were identified in 8 of 26 strains, with most effector alleles being >99% identical. Previous studies suggested Aux 3 was confined to clinical isolates, however Aux 3 was annotated in 7 environmental strains isolated during a 20-year period.

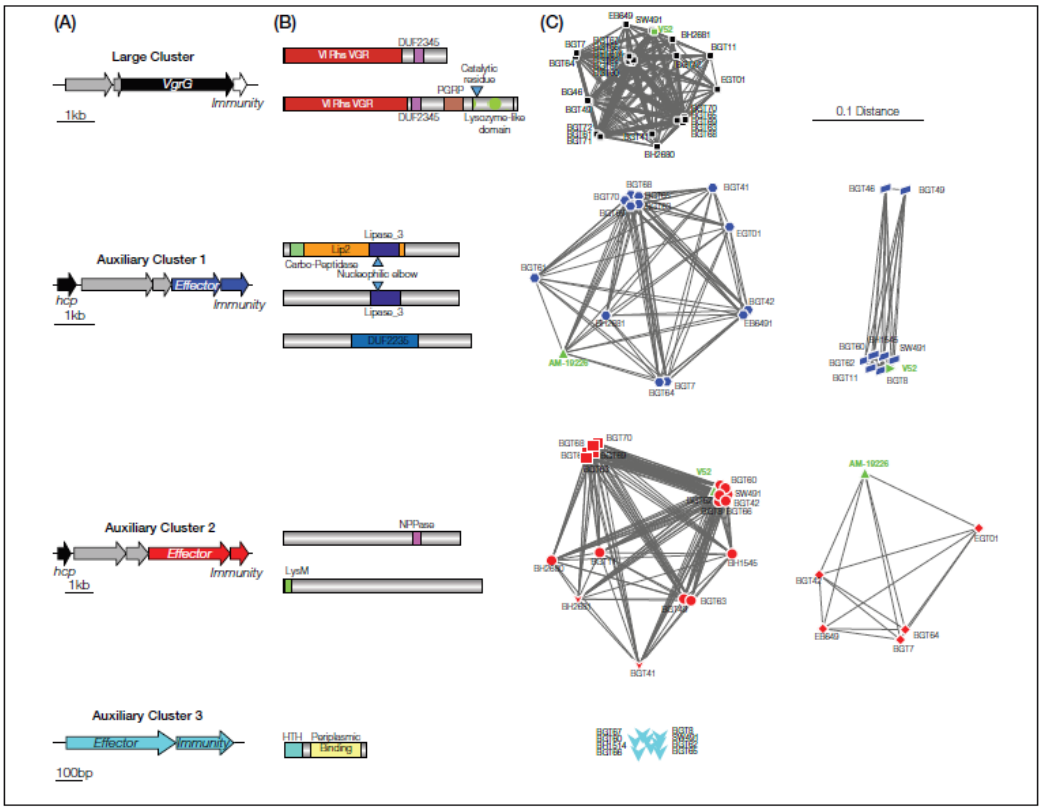


Figure 13. Previously described T6SS loci characterized in the *Vibrio cholerae* genomes sequenced in this study.

(A) All of the T6SS+ *Vibrio cholerae* genomes sequenced in this study contain one large cluster and two auxiliary clusters. The large cluster is located on chr2 and encodes the structural components of the T6SS (grey), a VgrG effector (black) and an immunity protein (white). Auxiliary clusters 1 and 2 are located on chr1 and chr2, respectively, and both encode a required structural protein, hcp (black), and effector/immunity protein pairs (blue and red, respectively). Auxiliary cluster 3 was found on chr2 in 8 of the 26 genomes analyzed here and encodes only for an effector/immunity pair (teal). (B) Schematic representation of domain structure of the effector proteins in each cluster is shown. (C) Self-organizing network maps of putative effector proteins is shown for each cluster. Each node represents the putative effector protein for a corresponding genome and the edge length represents the genomic distance between the pair. An edge is drawn only if the distance between the nodes is less than 20%. Aux 3 proteins are >99% identical, edges have been omitted for visualization purposes. The shape of the node denotes the characteristic domain: VgrG type 1 (black square), hydrolase (octagons), lipase (rhombus), transferase (red square), NTPase (circle), LysM (diamonds) and protein of unknown functions (arrow heads). Nodes that were characterized in previous studies are depicted as green triangles.

Effectors were typed and assigned to classes based on conserved structural and/or functional domains (Figure 13B). Domains were assigned using a custom database based on the Protein Families [170] and Conserved Domain [171, 172] databases. Best hit from rpsBLAST was considered for domain assignments, except in cases where a high scoring, complete superdomain was identified, but ranked below a subdomain; e.g., effectors containing the lipase_3 superdomain may have a higher scoring lip2 subdomain. Some domains – e.g. DUF2345, a T6SS associated domain – provide no functional information but aided in identification of true positive hits. Other domains – e.g. DUF2235, uncharacterized alpha/beta hydrolase domain – provide both putative function and confirmation of T6SS effector (DUF2235 containing T6SS proteins have been characterized in other bacteria, [187, 188]). Of note, conserved domains are not necessarily indicative of ultimate function. For example, hydrolase and lipase effectors likely have the same effect on target cells, degrading peptidoglycan. By contrast, a protein with a NPPase domain may simply insert the hydrophobic NPPase domain into target membrane in the host cell that promote cell function, and not target membranes in other cells that impair cell function and lead to cell lysis [62].

3.4.3 Hidden Markov Models for effector prediction and annotation of two new T6SS loci

HMMs are statistical models that relate a current state (or set of states) to the states that follow. When applied to genomic data, HMMs correlate sequence motifs and domain architecture with their likelihood of occurring in a particular order or position within a longer sequence. HMMer generated profile HMMs are similar to the position specific scoring matrices (PSSM) derived during psiBLAST, but make use of probabilities to

model subsequent sequence content instead of strict scoring rules. Much like PSSM scoring, HMMs profile sequences by windows, allowing for strong (statistically significant), partial matches of [sub] domains even when gaps or deletions are present. This fuzzy matching is useful when searching for related, but potentially distant proteins. Unlike psiBLAST PSSM's which are easily be poisoned by gaps and insertions, causing significant over or under fitting of the PSSM, HMMs are generally unaffected by gaps and insertions in the base multiple sequence alignment [189]. HMMs offer better biological significance between matches, more accurate predictions of evolutionary relatedness and homologue identification [190].

3.4.3.1 Aux 4

To investigate whether the isolates studied contain additional type 6 secretion system loci, the same set of genomes were interrogated with HMM models built for the genes of the Hcp and VgrG proteins. The hcp_degenerate model includes *impD* alleles, *and* an *hcp* homologue from plant pathogens such *Pseudomoas fluorescens* and human pathogens such as *Cronobacter sakazakii*. The *vgrG*_degenerate HMM was constructed using 20 computationally generated fragments of already identified *vgrG* alleles. Using the hcp_degenerate HMM, an additional *hcp*-like allele was identified in six environmental isolates (BGT46, 49, 61, 71, 72, EGT01). The vgrg_degenerate HMM identified an additional pseudo-vgrG in the same six isolates, in-frame and directly downstream of the *hcp*-like CDS. Each protein encoded by the gene directly downstream of the pseudo-*vgrG* contains a DUF4123 domain consistent with a T6SS adapter proteins (TAP). This newly identified cluster was annotated as Auxiliary cluster 4 (Aux 4), which is distinct in structure and content from Aux 3 and present in strains with an Aux 1 and 2 cluster. The

putative effector/immunity proteins do not match any previously reported *V. cholerae* effector/immunity proteins. Aux 4 effector has a conserved domain belonging to the AdoMet class (Figure 14A,B). A TMHMM prediction found no transmembrane helices with a high probability score for the effector to be non-cytoplasmic. This is consistent with other type six effectors that predominantly function in the periplasmic space of target cells [60]. A Phyre2 search for predicted protein folding yielded best hit matches with proteins from the ADC synthase superfamily, which in *E.coli* are known to catalyse the conversion of chorismate to deoxychorismate [191]. Further investigation is required to test whether this is a bona fide T6SS dependent effector.

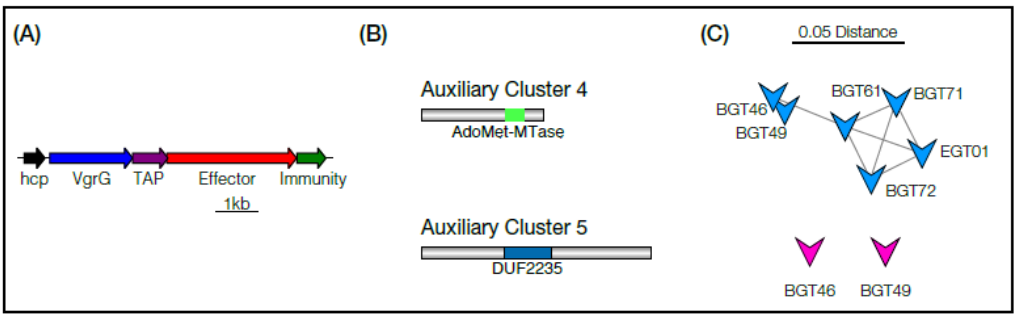


Figure 14. Novel T6SS clusters discovered in the *V. cholerae* genomes analyzed in this study.

(A) Two novel T6SS clusters, auxiliary clusters 4 (blue nodes) and 5 (pink nodes), were discovered in six and two strains respectively. Both clusters were found to exhibit the canonical auxiliary cluster arrangement of proteins: hcp (black), VgrG (blue), TAP (purple), Effector (red) and Immunity (green). (B) Schematic representation of domain structure of the effector protein in both clusters is shown. (C) Self-organizing network maps of putative effector proteins is shown for each cluster. Each node represents the putative effector protein for the indicated genome and the edge length represents the genomic distance between the pair. Aux 5 proteins are >99% identical and edges were omitted for visualization purposes.

3.4.3.2 Aux5

Additionally, the profile HMM built for Aux 1 DUF2235-containing effectors (hydrolases) identified a putative new T6SS locus in two related strains (BGT46, 49, Figure 14B). At time of initial identification, the upstream sequence containing hcp, VgrG and entire reading frame of the TAP was not known. Subsequent primer walking and Sanger sequencing have confirmed the presence of genes for an Hcp, VgrG, and (full length) TAP, confirming this locus is not an assembly artefact. This newly identified cluster is annotated as Auxiliary cluster 5 (Aux 5), and is distinct in content from Aux 1, 2, 3 and 4 and present in strains with Aux 1, 2, and 4 clusters.

3.4.4 T6SS effector networks

All-by-all comparison followed by visualization of the unrestricted effector network gave one large, highly interconnected network composed of several dense subgraphs connected by long edges. In an effort to better represent the evolutionary relationship within the network, an edge-weight cutoff was devised to remove spurious edges. Genes with the same annotation between genomes shared >95% identity in >99.99% of comparisons (Figure 25). The lowest observed identity value for a gene present in all 26 genomes was 22%. These findings along with Rost's twilight zone [177] of protein relatedness and evolutionary history suggested an edge-weight cutoff of 0.80 (1-Distance; 1.00-.20). Graphing the nodes after restriction revealed 8 clusters with no mixed graphs containing effectors from different loci (Figure 13C and Figure 14C). Each cluster corresponds to an effector type, the closest previously reported effectors were included

for Aux 1 and 2 (green triangles). Aux 3 and Aux 5 alleles are >99% identical, ~0 distance is represented by a cloud of nodes with no connecting edges.

3.4.5 T6SS Predictor: web tool for prediction of *V. cholerae* specific T6SS proteins

Finally, we introduce a tool for rapid prediction and annotation of putative T6SS loci and proteins. T6SS Predictor is a Shiny [192] application built in R [166] using custom Perl scripts to predict and annotate putative loci. T6SS Predictor takes as input either a protein FASTA file or genomic DNA FASTA file, with the option to provide a GFF annotation file instead of relying on *de novo* CDS prediction. Predictions take 2-5 minutes and the output is an annotated genome map of any identified loci and a FASTA File containing all the annotated, putative T6SS proteins.

CHAPTER 4. Horizontal gene transfer of functional Type Six Secretion System genes by chitin-induced natural transformation in *Vibrio cholerae*

4.1 Abstract

In many strains of the waterborne pathogen *Vibrio cholerae*, chitin induces expression of genes for a competence apparatus, which allows for uptake of extracellular DNA that can recombine onto the chromosome by homologous recombination and promote natural transformation. Often the competence apparatus is also co-expressed with a type VI secretion system (T6SS) for engaging in contact-dependent antagonism against adjacent cells. All sequenced *V. cholerae* strains code for at least three T6 loci: a large gene cluster encoding conserved structural components and two auxiliary clusters (Aux 1 and 2). A diverse effector protein and adjacent cognate immunity protein to prevent self-intoxication are encoded at the end of each cluster, and predicted to be horizontally transferred between *Vibrios* based on genomic analyses. Previously we identified a transformation-proficient clinical strain and transformation-deficient environmental strain with distinct Aux 1 and 2 effector-immunity pairs that engage in mutually antagonistic T6-mediated interactions. Here we show that 1) the clinical strain can acquire T6 auxiliary cluster DNA from the environmental strain in co-culture on chitin, 2) the acquired T6 loci are functional in the new genetic background, and 3) the transformants engage in T6-killing distinct from either parental strain. This is the first *in vivo* demonstration of horizontal gene transfer of type VI secretion system genes via natural

transformation and highlights the role of transformation as a mechanism to promote diversity in this important human pathogen.

4.2 Introduction

25% of all sequenced Gram negative bacteria carry genes for a Type VI Secretion System (T6SS), an apparatus capable of puncturing and delivering toxins into both eukaryotic and bacterial cells [53] The widespread occurrence of this contact- dependent toxin delivery system in bacteria suggests that T6SS might be commonly employed to exclude competitors growing in close proximity in environmental or host-associated settings. The human pathogen *Vibrio cholerae* utilizes the T6SS in aquatic settings and in the human intestine to deliver into neighboring cells toxic effectors that result in cell lysis and death [102, 193].

In *V. cholerae* T6SS genes are organized into several distinct loci including a large cluster that encodes the majority of the structural genes, and at least two auxiliary clusters [58], which each express a toxic effector that can be delivered into neighboring cells using the T6SS apparatus [62]. Self-intoxication is mitigated by expression of a cognate immunity protein that is often encoded directly downstream of the effector protein and is transcribed from a promoter embedded within the coding sequence of the upstream effector [60-62]

T6SS$^+$ *V. cholerae* can cause contact-dependent death of a number of gram negative bacteria, and of isogenic *V. cholerae* lacking immunity proteins, and can compete with other T6SS$^+$ *V. cholerae* expressing distinct classes of effector/immunity pairs [56, 62]. The diverse repertoire of toxic effector proteins include lipases, nucleases,

hydrolases and peptidoglycan degrading enzymes [62]. Typically clinically-derived, toxigenic *V. cholerae* isolates like reference strain C6706 control expression of T6SS genes with a regulatory network comprised of several transcription factors that are induced in response to external chemical signals. For example, presence of chitin leads to up-regulation of transcriptional regulator TfoX which activates T6SS gene expression [84, 152] while accumulation of auto-inducers at high-cell density leads to activation of quorum sensing regulator HapR which also up-regulates T6SS gene expression [84, 152]. In addition, CytR, a nucleoside scavenging response regulator which is activated under starvation conditions, was shown to be required for T6SS activation on chitin [152]. These three regulators converge to activate expression of transcriptional regulator QstR which is required for T6SS activation in C6706 [152]. By contrast, isolates of environmental origin like 692-79 express T6SS activity in the absence of chitin and can constitutively kill prey *E. coli* in lab conditions [4]. Evidence suggests that such isolates utilize a distinct regulatory pathway requiring the TfoY regulator. [83] The regulatory network controlling expression of the T6SS on chitin in *Vibrio cholerae* also controls expression of the competence machinery required for DNA uptake and natural transformation [84, 152]. Thus *Vibrio cholerae* co-ordinately expresses a T6SS machinery used to kill neighboring cells as well as a DNA uptake apparatus which is capable of taking up DNA released from neighboring cells that are lysed due to T6SS mediated antagonism [84].

Bioinformatics evidence suggests that T6SS loci might be shared horizontally amongst *Vibrios* and are often found in genomic neighborhoods containing mobile genetic elements [194], (Chande et al, unpublished). Similarly, horizontal exchange of

diverse effector-immunity pairs via homologous recombination has also been proposed based on differences in G-C content and sequence identity observed between T6SS Auxiliary clusters of different strains [62, 65] .

Here we show that novel T6SS effector-immunity proteins can be acquired by *Vibrio cholerae* via chitin-induced natural transformation with naked donor DNA and when a donor and recipient are incubated in co-culture. “Hybrid” transformants with newly acquired T6SS effectors are proficient at killing clinical recipient cells, but show decreased antagonism towards the environmental donor. Similarly, the hybrid transformants are greatly protected against T6SS attacks by the environmental donors, but show increased susceptibility to attacks by the clinical recipient. This suggests the intriguing possibility that *V. cholerae* may be capable of continuous adaptation to different microbial challengers by rapid horizontal acquisition of new molecular weaponry via transformation.

4.3 Results

4.3.1 Polymorphism in Type six secretion system effector modules

Among a set of environmental *V. cholerae* isolates we recently sequenced (Watve, et al 2016), Bernardy et al identified two “mutual killer” strains that engage in antagonism with each other in a T6SS-dependent manner [195]. To test whether the incompatibility between these strains could be due to differences in their T6SS effector-immunity modules, we performed pairwise nucleotide alignments for each of the known T6SS clusters between the non-toxigenic environmental isolate, 692-79 and the toxigenic clinical reference isolate, C6706 (Figure 26 and Figure 15). Comparisons between Aux 1

regions for both strains revealed a high degree of conservation (98% nucleotide identity) between the N-termini of VgrG-1 proteins. By contrast, C6706 contains a C-terminal actin cross-linking domain toxic against eukaryotic cells [54], while 692-79 lacks this domain. Both strains also showed differences (46.2% nucleotide identity) in the region encoding Tap proteins which are thought to chaperone effectors onto the T6S apparatus by associating with VgrG proteins [65, 66](Figure 15). As expected, these mutual killer strains harbor distinct effector-immunity pairs. The effector protein of C6076 Aux1 is an antibacterial lipase TseL [60] while the effector of 692-79 is predicted to be a DUF2235-containing phospholipase similar to Tle1 from *Pseudomonas aeruginosa*. Differences were also observed for Aux 2, notably with both strains encoding effectors that fall into distinct classes (Figure 15) (T6SS effector networks). Cognate immunity proteins are tailored to prevent effector toxicity by a variety of mechanisms and as expected show differences amongst these two strains at the nucleotide level for both Auxiliary clusters 1 and 2 (Figure 15). Finally, genes encoded downstream of both auxiliary clusters showed >98% nucleotide identity indicating high conservation across strains. Differences between the variable c-termini of VgrG3 proteins encoded in the main cluster as well as the cognate immunity protein were also observed (Figure 26). This pattern of polymorphism of effector modules in an otherwise highly conserved gene neighborhood is consistent with previous reports and has led to the hypothesis that effector-immunity modules are frequently exchanged by horizontal transfer amongst different isolates[62, 65]

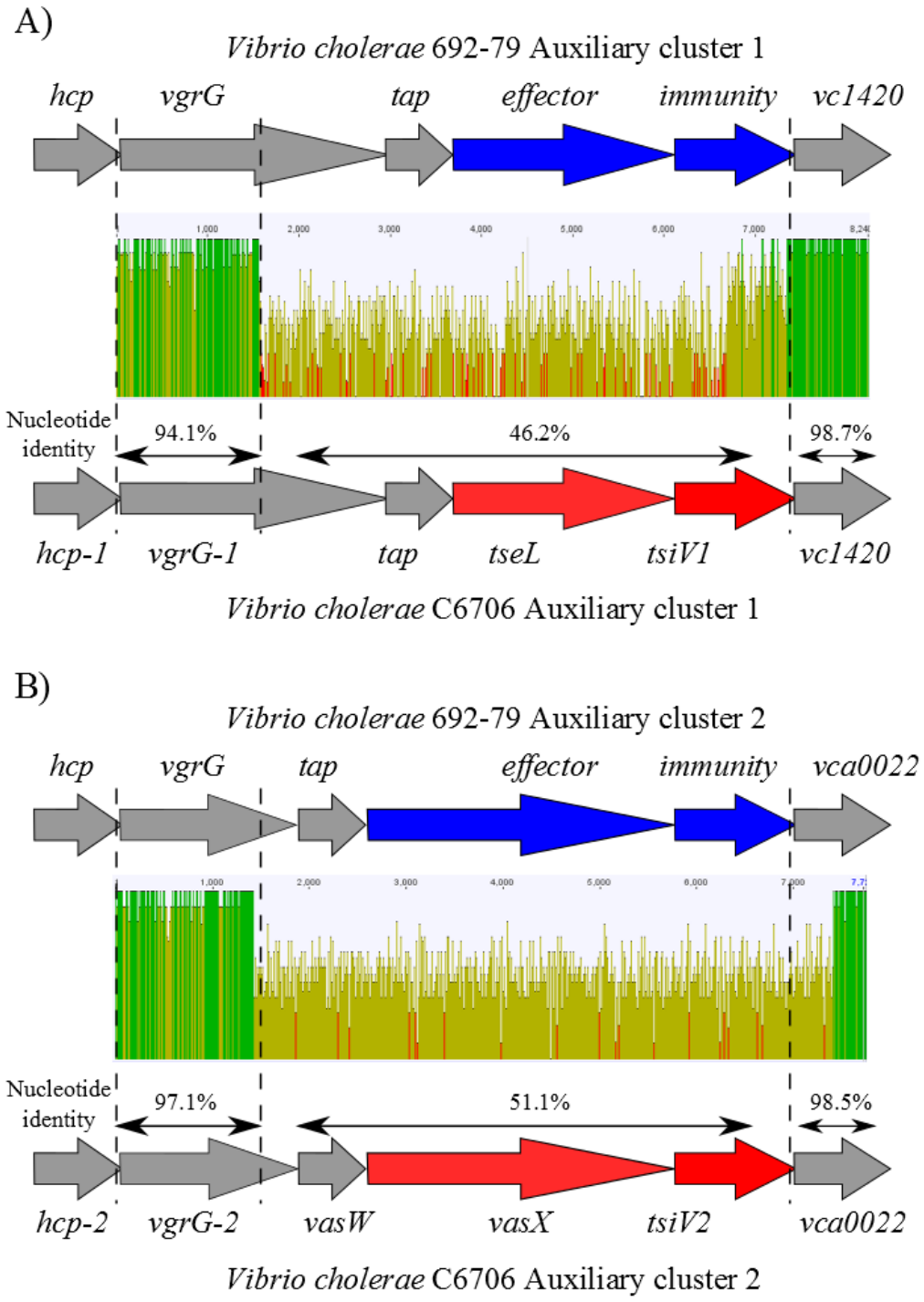


Figure 15. Polymorphism in type six secretion system effector modules

Pairwise nucleotide alignments were performed between type six secretion system A) Auxiliary cluster 1 and B) Auxiliary cluster 2 from an environmental (non-toxicogenic) isolate 692-79 and clinical (toxicogenic) isolate C6706. Both clusters show low sequence conservation in the effector/immunity loci (middle) flanked by high conservation upstream and downstream indicating that the two sequences might have distinct origins.

4.3.2 Horizontal gene transfer of type six secretion system genes

Natural transformation is one of the primary modes of horizontal gene transfer in bacteria. Because many clinical isolates of *V. cholerae* coordinately expresses both the competence apparatus for DNA uptake and the T6SS on chitin, we sought to test whether T6SS genes can be horizontally acquired via chitin-induced DNA uptake. To this end we constructed Kanamycin resistant (KanR) derivatives of environmental isolate 692-79, as well as an isogenic ΔvasK mutant deficient for T6SS killing activity, by integrating a *kan* cassette downstream of both Aux cluster 1 and 2 (see materials and methods). These KanR derivatives of 692-79 served as donors in subsequent experiments, and allowed putative T6SS "hybrid" transformants that acquire a novel T6SS cluster via transformation to be detected by genetic linkage with the selectable marker. Genomic DNA isolated from these strains served as eDNA for initial transformation experiments [119]. C6706 (see strain list) and an isogenic Δ*vasK* mutant, were both transformable and acquired Aux 1 and Aux 2 DNA at similar frequencies (Figure 16A). We refer here to the transformants thus obtained as "Aux1 hybrid" and "Aux2 hybrid", respectively. Horizontal acquisition of T6SS clusters was dependent on the competence machinery of *V. cholerae*, because no KanR transformants were obtained for either C6706 Δ*comEA* or C6706 Δ*vasK* Δ*comEA* mutants which were incapable of DNA uptake (Figure 16A).

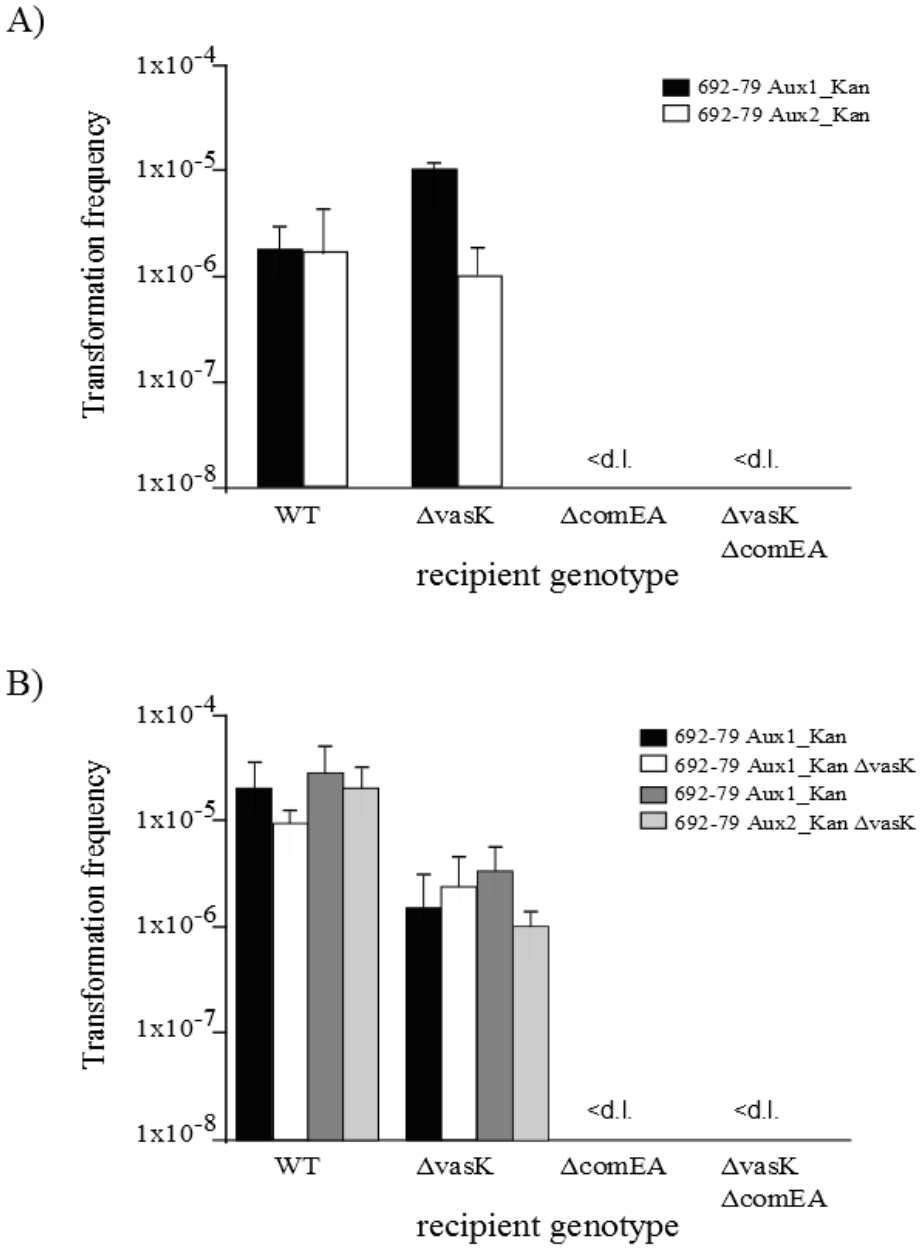


Figure 16. Horizontal transfer of T6SS genes via chitin induced natural transformation.

A) A *V. cholerae* C6706 isolate and an isogenic Δ*vasK* derivative undergo transformation when exposed to genomic DNA from 692-79, containing a KanR cassette downstream of Aux cluster 1 (black bars) or Aux cluster 2 (white bars). No KanR transformants were ever observed for the C6706 Δ*comEA* or C6706 Δ*vasK* Δ*comEA* mutants. B) WT and Δ*vasK* also undergo transformation when grown in co-culture with 692-79 isolates

containing containing a KanR cassette downstream of Aux cluster 1 (black bars) or Aux cluster 2 (light grey bars) or 692-79 isogenic Δ*vasK* isolates containing a KanR cassette downstream of Aux cluster 1 (white bars) or Aux cluster 2 (dark grey bars) respectively. Transformation frequency is calculated as KanR cfu.ml^{-1}/Total cfu.mL^{-1}. The limit of detection is 1 x 10^{-8} cfu mL^{-1}. Data are shown as mean ± sd. All data are from six independent biological replicates.

While naked extracellular DNA can facilitate natural transformation in lab mesocosms, in natural settings, donor DNA is liberated by lysis of bacteria in natural consortia [6]. Thus we reasoned HGT of T6 genes could occur between co-cultured *V. cholerae* strains that mimic consortia found on chitinous surfaces. Because *V. cholerae* 692-79 is non-transformable, unlike chitin-inducible C6076 [4], we engineered a C6706-specR derivative and isogenic Δ*vasK* mutant to serve as recipients and co-cultured with the 692-79-KanR donor and isogenic Δ*vasK* mutant on crab shells followed by selection of transformants on medium containing both kanamycin and spectinomycin. No KanR transformants were obtained for either C6706 Δ*comEA* or the Δ*vasK* Δ*comEA* mutants in co-culture. We observed ~10-fold higher transformation frequencies with WT C6706 recipient as compared to the isogenic Δ*vasK* recipient, irrespective of the T6SS status of the donor strain (Figure 16B). This difference can be attributed to differences in killing ability of the two strains. T6SS^{+} WT C6706 that activate DNA uptake and T6SS on chitin can lyse recipient cells making more DNA available for uptake, while the T6SS^{-} Δ*vasK* C6706 mutant cannot lyse recipients but only take up DNA released by spontaneous cell lysis. Thus, transformation proficient *Vibrio cholerae* cells can acquire T6SS alleles from a competitor while growing in close proximity with the competitor on a chitinous surface.

4.3.3 Horizontally acquired effectors replace corresponding recipient loci by homologous recombination

Natural transformation in *V. cholerae* and other bacteria requires the recombinase protein RecA for incoming eDNA to be incorporated into the chromosome by homologous recombination [196]. Successful incorporation of novel T6SS clusters via natural transformation leads to replacement of native T6SS effector/immunity alleles in the recipient with those of the donor (Figure 26), and thus likely affects compatibility of T6SS hybrids with parental strains: the clinical C6706 recipient or the environmental 692-79 donor.

4.3.4 Novel effectors can be used for T6SS killing of parental cells

Next we performed killing assays with the hybrid transformants as predators against a C6706 Δ*vasK* prey in order to determine the toxicity of newly acquired effector cassettes against parental C6706 donor. We saw no difference in prey survival while competing C6706 Δ*vasK* prey with either WT or Δ*vasK* predators, since cells with identical effector/immunity proteins do not kill one another (Figure 17A). However we observed ~10-100-fold prey killing by the Aux1 hybrid transformant compared to an Aux 1 Δ*vasK* killer. Similarly, we observed 100-1000-fold prey killing by the Aux2 hybrid transformants compared to an Aux 2 Δ*vasK* killer (Figure 17A). Additionally we obtained an Aux1 + 2 double hybrid by performing chitin-induced transformation of the Aux1 hybrid strain obtained previously with 692-79 genomic eDNA marked with an antibiotic resistance cassette downstream of Auxiliary cluster 2. To see if the Aux1 + 2 double hybrid can kill parental cells in a T6SS dependent manner, we constructed an Aux1 + 2 hybrid Δ*vasK* strain and competed it against C6706 Δ*vasK* prey. We observed ~2-3 logs of prey killing by the Aux1 + 2 hybrid compared to an isogenic Δ*vasK* killer, similar to killing obtained by an Aux 2 hybrid strain (Figure 17A). Similarly, we also

investigated survival of C6706 Δ*vasK* against different hybrid competitors via fluorescence microscopy by constructing a derivative of C6706 Δ*vasK* that constitutively expresses green fluorescent protein GFP mTFP1 and competing against the hybrids that constitutively express RFP mKO. All three T6SS hybrid strains outcompeted C6706 (Figure 17B) when they were T6SS proficient, while stable mixtures of both green and red cells were obtained when the hybrids were T6SS deficient (Δ*vasK*). This indicates that newly acquired effectors are functional in the new genetic background and can be used as potent weapons in competition against parental cells. We initially expected the Aux1+2 hybrid predator to be a better killer than the Aux2 hybrid, but we did not observe major differences in their killing ability against C6706. There are several models that might explain this discrepancy. First, toxic effects of different effectors may not be independent and additive and may interact co-operatively or antagonistically depending on the context. Second, efficient secretion of toxins requires proper assembly of the T6SS apparatus and presence of multiple horizontally acquired T6SS components may hinder formation of fully functional T6SS complexes and might therefore affect overall killing efficiency.

Next we wanted to test whether loss of parental effectors via homologous recombination negatively impacted the ability of Aux cluster hybrids to kill the environmental isolate. We performed killing assays with the same set of predators against a 692-79 Δ*vasK* prey. WT C6706 was the best killer with ~5 logs of killing activity, which decreased with each subsequent loss of original effector alleles (Figure 17C). Both Aux1 hybrid and Aux2 hybrid strains showed a decreased killing ability (~2-3 logs) while the Aux1+2 double hybrid showed only 1-2 logs of killing (Figure 17C). This

shows that acquisition of T6SS alleles by transformation may lead to gene loss that decreases the ability to kill competing cells. We also competed GFP expressing 692-79 Δ*vasK* cells against T6SS hybrids and observed results similar to those obtained with C6706. All three T6SS hybrid strains outcompeted 692-79 (Figure 17D) when they were T6SS proficient, while stable mixtures of both green and red cells were obtained when the hybrids were T6SS deficient (Δ*vasK*).

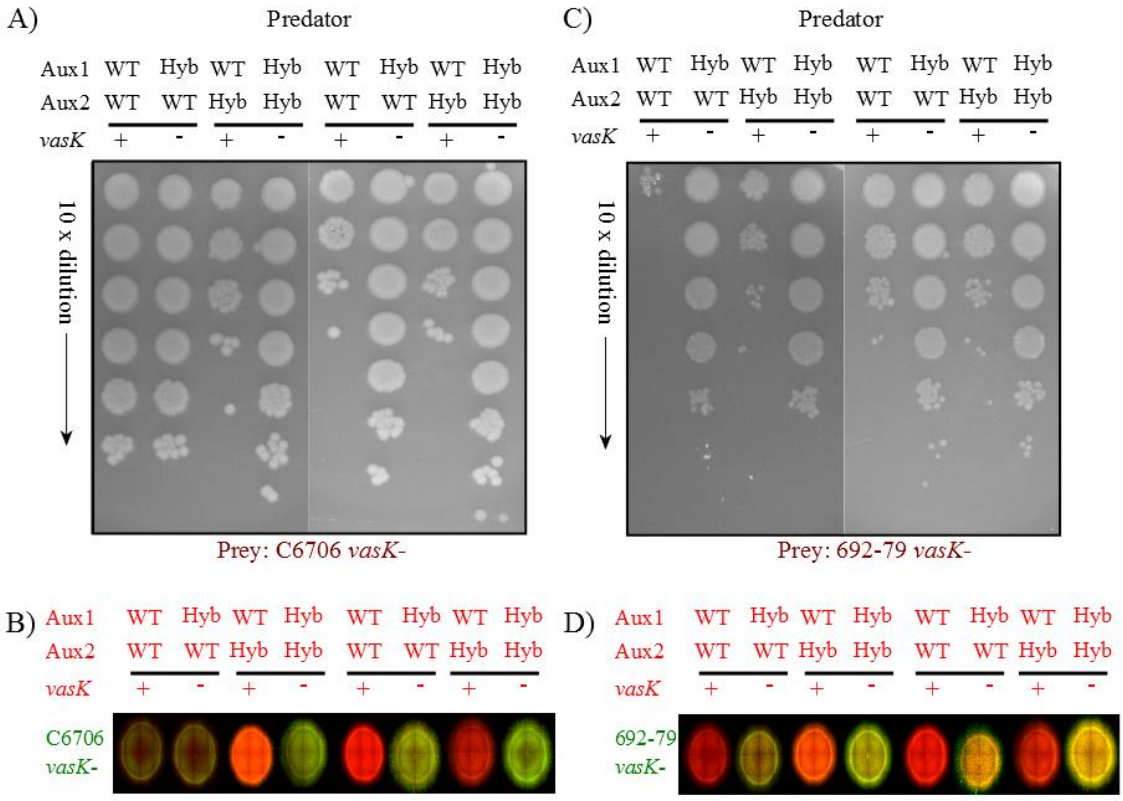


Figure 17. Novel effectors are toxic against C6706 prey

Hybrids are proficient for T6SS attacks against C6706 Δ*vasK* prey. A) 100-1000 fold decrease in surviving cfu of C6706 Δ*vasK* prey is observed when competing against the Aux1, Aux2 or Aux1+2 hybrid predators but not against corresponding Δ*vasK* predators. B) RFP (mKO) labelled Aux1, Aux2 or Aux1+2 hybrid predators outcompete GFP (mTFP1) labelled C6706 Δ*vasK* at a 1:1 ratio after 24 hours of co-incubation at 30°C. Hybrids are deficient for T6SS attacks against 692-79 Δ*vasK* prey. C) 100-1000 fold increase in surviving cfu of 692-79 Δ*vasK* prey is observed when competing against the

Aux1, Aux2 or Aux1+2 hybrid predators while Δ*vasK* predators no killing. D) 692-79 Δ*vasK* prey are outcompeted by T6SS proficient C6706 cells or Aux1, Aux2 or Aux1+2 hybrid cells but can stably co-exist with T6SS deficient cells when incubated at a 1:1 ratio for 24 hours at 30°C. All experiments were performed in triplicate but representative images are shown here.

4.3.5 Novel immunity proteins protect against T6SS killing by environmental isolate 692-79

We also tested whether replacement of original immunity alleles had similar consequences on surviving T6SS attacks from parental C6706 and environmental cells. We competed 692-79, 692-79 Δ*vasK*, WT and Δ*vasK* predators against Δ*vasK* versions of the hybrid isolates as prey (Figure 18A and C). *Vibrio cholerae* 692-79 was proficient at killing WT C6706 prey cells and we observed ~4 logs decrease in survival of WT C6706 prey cells as compared to 692-79 Δ*vasK* predator (Figure 18 A). Aux1 hybrid prey was also killed ~4 logs by WT 692-79 predator as compared to the 692-79 Δ*vasK* predator (Figure 18A), while the Aux2 hybrid and Aux1 + 2 double hybrid showed improved survival and were killed ~3 logs and ~2 logs respectively as compared to the 692-79 Δ*vasK* predator (Figure 18A) indicating that acquisition of novel immunity factors provides a survival benefit to hybrid cells. The opposite pattern was observed for prey survival against WT C6706 predator cells. WT C6706 prey cannot be killed by WT cells since they are isogenic for effector/immunity pairs (Figure 18C), while the Aux1 hybrid, Aux2 hybrid and Aux1 hybrid prey are susceptible to T6SS attacks by WT C6706 cells and show ~2 logs,~3 logs and ~3 logs decreased survival respectively against WT C6706 predator cells (Figure 18C). Thus loss of immunity proteins increases susceptibility to T6SS attacks by parental cells. Fluorescence microscopy revealed that T6SS proficient hybrids still out-competed environmental 692-79 cells (Figure 18B) even though they too were T6SS proficient and capable of executing T6SS attacks against the hybrid isolates.

This may be due to presence of additional toxic effectors that C6706 employs against 692-79 cells that were not exchanged by horizontal transfer or due to differences in growth rate that allow hybrid cells to emerge victorious over time. Interestingly, the Aux1 hybrid Δ*vasK* strain was almost completely eliminated by T6SS proficient 692-79 except for a small number of micro-colonies of red Aux1 hybrid cells (Figure 18B). A previous study have showed that T6SS mediated antagonism between mutual killers in a well-mixed population can lead to phase separation [195]. However in that study phase separation was obtained when both strains were capable of killing. In this case one strain (692-79) is killing proficient, while the other (Aux1 hybrid) is deficient for killing and only survives via immunity against T6SS attacks. Indeed, greater levels of survival (and phase separation) were obtained for the Aux2 hybrid Δ*vasK* strain with the highest survival for Aux1+2 hybrid Δ*vasK* strain which has two immunity proteins expressed by 692-79 cells (Figure 18B).

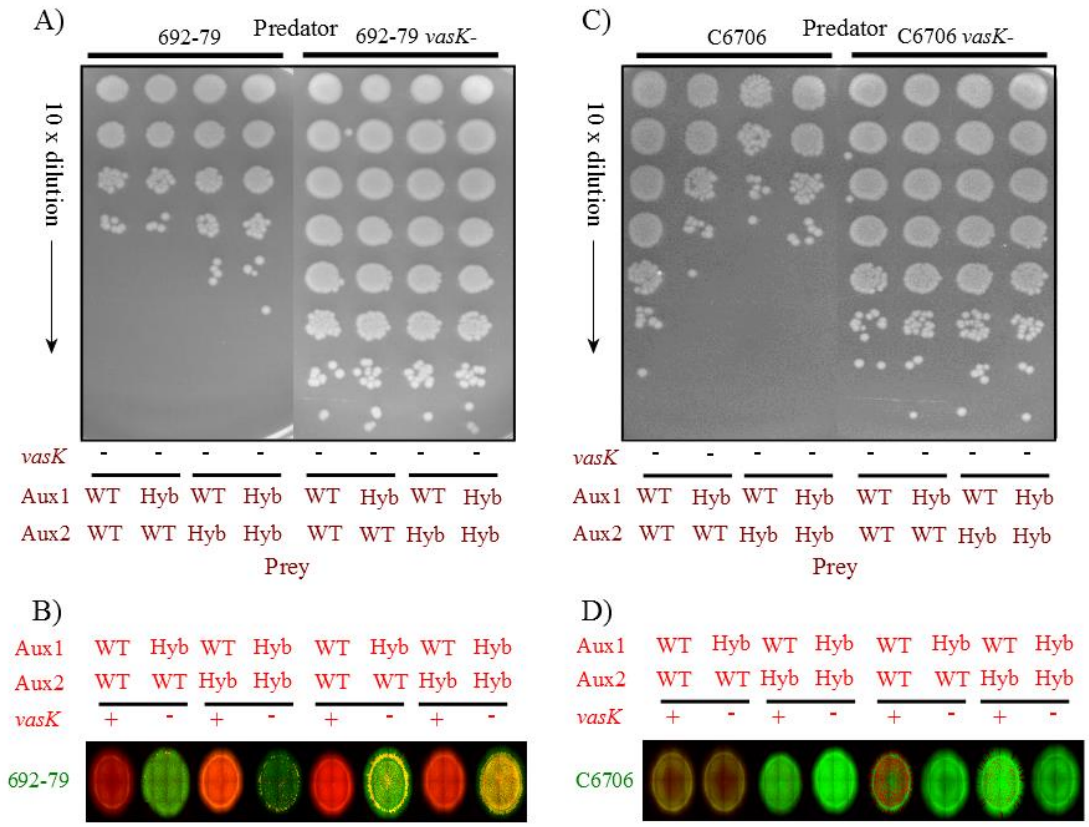


Figure 18. Novel immunity proteins confer protection against T6SS attacks by 692-79

Hybrids are less susceptible to T6SS attacks from a 692-79 predator. A) 100-1000 fold increase in surviving cfu of C6706 Δ*vasK* prey is observed when competing the Aux1, Aux2 or Aux1+2 hybrid prey relative to the C6706 prey against a 692-79 predator but not against corresponding Δ*vasK* predators. B) RFP (mKO) labelled C6706, Aux1, Aux2 or Aux1+2 hybrid predators outcompete 692-79 green (mTFP1) predator at a 1:1 ratio after 24 hours of co-incubation at 30°C. However, T6SS- C6706 lose against 692-79, while increased survival is observed for Aux1, Aux2 and Aux1+2 hybrids. C) 100-1000 fold decrease in surviving cfu of Aux1, Aux2 or Aux1+2 hybrid prey is observed when competing against C6706 while Δ*vasK* predators show no killing. D) C6706 WT (green) can outcompete Aux1 and Aux1+2 hybrid (red) and stably co-exist with an Aux2 hybrid (red) when incubated at a 1:1 ratio for 24 hours at 30°C. T6SS- hybrids are susceptible to killing by WT C6706 cells. All experiments were performed in triplicate but representative images are shown here.

This pattern of survival is consistent with improved survival of Aux2 and Aux1+2 hybrid obtained in killing assays above. Thus, immunity against T6SS attacks can also lead to stable co-existence via phase separation in a spatially constrained environment. Lastly, we observed no survival for any of the hybrid Δ*vasK* strains against C6706 (green) (Figure 18D) by fluorescence microscopy. However T6SS proficient hybrids showed increased survival with a few micro colonies observed for the Aux1 hybrid and Aux1+2 hybrid with the greatest survival for the Aux2 hybrid (Figure 18D), once again suggesting that different T6SS alleles interact in complex ways.

4.3.6 Killing of other clinical isolates

Previous reports identified several toxicogenic strains that harbor highly similar effector immunity proteins including *Vibrio cholerae* CA401 which has the O1 classical serogroup and *Vibrio cholerae* MO10 from the O139 ElTor serogroup that have similar effector-immunity protein to that of C6706[62]. In order to test whether the hybrid isolates were capable of killing *Vibrio cholerae* CA401 and MO10 we performed killing assays as described. While WT C6706 predator was incapable of killing CA401 or MO10 100-1000 fold fewer cfu's were recovered when competed with the Aux1, Aux2 or the Aux1+2 double hybrids (Figure 27), indicating that the hybrids had gained the ability to compete with other clinical isolates using T6SS dependent attacks.

4.4 Discussion

Why bacteria take up DNA and undergo recombination given the possibility of accumulation of deleterious mutations, has been a long-standing puzzle in bacterial genetics [197-199]. The modular architecture of T6SS effector-immunity pairs, coupled

with their overall diversity as well as unique sequence characteristics within the tap proteins have prompted others to suggest that T6SS alleles might be frequently exchanged via horizontal transfer [62, 65]. We have demonstrated that *Vibrio cholerae* can horizontally acquire novel T6SS effector-immunity coding genes via chitin-induced natural transformation by taking up DNA from a related *Vibrio cholerae* isolate. Upon acquisition of novel T6SS effector genes, they are expressed in the novel genetic background and are translocated in a T6SS dependent manner and can be used in T6SS attacks against other cells. Based on these observations we conclude that acquisition of novel effector/immunity pairs via natural transformation is a double-edged sword. On one hand, acquiring novel effector/immunity pairs increases killing ability against parental cells and provides immunity against T6SS attacks by competing environmental cells, while on the other hand loss of original effector immunity pairs leads to increased susceptibility to predation by sister cells that are isogenic except for the newly acquired locus and simultaneously decreases the ability to kill competing environmental cells. Additionally, the change in magnitude of killing ability or susceptibility to T6SS attacks is allele-specific. Exchanging of the Aux2 cluster has a ~10-fold greater impact on the ability to kill parental cells as well as escaping predation by environmental cells (Figure 17A and Figure 18A) compared to an Aux1 exchange. However, while both Aux1 and Aux2 hybrids kill 692-79 cells to similar degrees (Figure 17C), the Aux2 hybrid has ~10 fold greater susceptibility to T6SS attacks by the parental cells (Figure 18C) compared to the Aux1 hybrid. Additionally the Aux2 hybrid is able to better compete against T6SS proficient C6706 cells (Figure 18D). Similarly, exchange of native T6SS alleles with alleles from an environmental isolate provided Hybrid isolates with the ability to

outcompete several clinical isolates that was absent in the parent, suggesting that hybrid isolates might be better suited to compete with clinical isolates. Interestingly, a previous study showed that clinical (toxicogenic) isolates largely share the same sets of effector-immunity proteins [62]even though several clinical isolates are known to be naturally transformable. This apparent lack of diversity in effector-immunity subtypes within clinical may be due to a sampling bias towards clinical isolates that are very closely related to each other[146]. Alternatively, T6SS in *Vibrio cholerae* may play a less critical role for survival in the human host and transmission for person to person[82]. However, recent studies suggests that T6SS is active inside host guts [101] and can be induced by host factors that mimic chitin such as mucin [65]. Thus it is also possible that a specific combination of T6SS effector immunity alleles contained within clinical isolates is optimal for surviving inside the human host and effectively competing against resident microflora.

4.5 Materials and Methods

4.5.1 Bacterial strains, plasmids, and culture conditions.

All *V. cholerae* strains were derivatives of a streptomycin resistant C6706 El Tor biotype O1 clinical strain (BH1514) or environmental strain 692-79 (BGT64) (Table S1, supplemental material). Bacteria were commonly grown at 37°C in Luria broth (LB) under constant shaking, or statically on petri plates containing LB agar, supplemented with ampicillin (100 µg/mL), kanamycin (50 µg/mL), chloramphenicol (10 µg/mL for *V. cholerae* and 25 µg/mL for *E.coli*), diaminopimelic acid (DAP 50 µg/mL), and streptomycin (5 mg/mL) where appropriate.

4.5.2 Recombinant DNA techniques.

Standard molecular biology-based methods were utilized for DNA manipulations. DNA modifying enzymes and restriction nucleases (Promega and New England Biolabs), Gibson assembly mix (New England Biolabs), Phusion DNA Polymerase (New England Biolabs), and Taq DNA polymerase (Promega) were used following the manufacturer's instructions. All modified DNA fragments were tested by colony PCR and verified by Sanger sequencing (Eurofins).

4.5.3 Bioinformatics.

Plots for pairwise nucleotide alignments between the two isolates were obtained using Geneious v8.1.7.

4.5.4 Transformation assays.

Transformation assays on chitin as well as co-culture experiments were performed as described [119]. KanR Aux1 hybrids were scored for presence of horizontally acquired effector protein from 692-79 and concomitant loss of TseL from C6706 by allele-specific multiplex PCR as shown in Suppl fig (xxx) with primers GT2100 (5'-gaagagacagctaaaccggttgaagag-3'), GT2101 (5'-gttaattcattacttgcactacccgcaac-3'), GT2104(5'-tgcagtgtaaccctgaggaaaactg-3'), GT2105(5'-ttgctatatggaatttcctctaataaccactttgc-3'). Similarly, KanR Aux2 hybrids were scored for presence of horizontally acquired effector protein from 692-79 and concomitant loss of TseL from C6706 by allele-specific multiplex PCR Transformation frequency using

primers GT2077(5'-acgctgacatattgtatgtgccc-3'), GT2078(5'-taattttgtcgataaccaaagctcgtaactgg-3'), GT2092(5'-tcaccctcatgcacattttgctca-3'), GT2093(5'-gagctcggtgtaatgctccac-3'). Presence of a 500bp band on an agarose gel indicated a T6SS hybrid while a 700bp band indicated WT effector allele was intact. Transformation frequency was defined as KanR cfu mL−1 x %T6SS hybrids/total cfu mL−1.

4.5.5 Construction of genetically modified strains of *Vibrio cholerae.*

In-frame deletions in *V. cholerae* C6706 were constructed by allelic exchange using pKAS32 [42] or pRE118-based plasmids [200]. pRE118 was a gift from Dieter Schifferli (Addgene plasmid # 43830).

4.5.6 T6SS killing assay.

The T6SS killing assay was modified from previously described methods [56]. *V. cholerae* strains grown overnight in LB medium at 37°C were pelleted by centrifugation and re-suspended in fresh LB medium to an OD600 of 1.00. Predator and prey strains were mixed at a ratio of 10:1 and 50 µL of each suspension was spotted onto each well of a 12-well sterile microtiter plates (Corning) containing 2 mL of LB agar. After incubation at 30°C for 24 hours, cells were re-suspended in LB medium and dilutions were spotted onto LB agar supplemented with appropriate antibiotic to determine prey survival.

4.5.7 Microscopy.

Overnight cultures incubated with shaking at 30C were normalized for OD600, mixed at a 1:1 ratio and and 1 µl was inoculated onto LB agar (1.5%) pads on glass slides, and incubated at 30 C for 24 h. Confocal Laser Scanning Microscopy (CLSM) was performed on a Nikon A1R system using filters FITC (for mTFP1, cyan) and TRITC (for mKO,

orange). Full colony images were captured in one z-plane using a 10x CFI Plan Apochromat objective lens and 512 X 512 pixel images were captured by Galvano scanning. For every sample, the top and bottom of the colony was located, and a plane in the middle was imaged. The images were stitched and channels merged using NIS Elements software.

CHAPTER 5. Conclusions and recommendations

The ability to sense and respond to environmental signals is crucial for the survival and adaptation of bacteria. The aquatic environment that serves as the natural habitat for *Vibrio cholerae*, serves as a reservoir for future epidemic outbreaks. Thus it is crucial to understand the mechanisms by which *V. cholerae* survives and persists in diverse environments. Some of the adaptations that aid *V. cholerae* in survival and persistence include the ability to utilize chitin which is an abundant biopolymer, genetic competence for natural transformation and the Type VI secretion system (T6SS) used for bacterial killing. All three phenotypes are regulated by a common regulatory mechanism involving three extracellular signals and associated transcriptional regulators: chitin (TfoX), quorum sensing autoinducer molecules (HapR and QstR), and extracellular nucleosides (CytR), deeper understanding of this regulatory scheme is important for insights into the ecology and evolution of this pathogen. However, while transformation and T6SS are coordinately expressed and utilized in clinical isolates commonly studied in laboratories, expanding our understanding to poorly characterized isolates from environmental sources is equally important since they appear to employ different regulatory schemes.

The research detailed in this dissertation aimed to dissect the regulatory scheme controlling natural competence and T6SS-mediated bacterial killing among various clinical and environmental isolates of *V. cholerae* as well as investigate the evolutionary and ecological consequences of horizontal transfer and T6SS killing in bacterial communities. To this end, I performed RNA-Seq experiments in *V. cholerae* C6706 to

uncover the TfoX and CytR regulon. CytR was only studied prior as a regulator of nucleoside scavenging in *E. coli* and biofilm formation in *V. cholerae*. Second, I sequenced a set of environmental and clinical isolates collected over the past 100 years that were recently characterized for their chitinase activity, transformation proficiency and constitutive T6SS killing. We developed a pipeline to identify and classify novel T6SS effectors and were able to identify two entirely novel clusters that had not been reported earlier. Third, I documented horizontal transfer of T6SS genes from an environmental isolate to a transformation proficient clinical isolate C6706. These two strains can mutually kill each other by using their T6SSs and I showed that acquiring novel T6SS clusters altered their antagonistic interactions. We discovered that transformation of T6SS loci leads to allele replacement of existing effector immunity pairs. While hybrids strains gain proficiency at killing parental (recipient) clinical cells, their ability to kill environmental (donor) cells decreases. Similarly hybrid strains are protected from killing by the competitor, but are susceptible to T6SS attacks from clinical siblings.

The results of this study suggest several areas of future research:

- Many regulatory components that activate natural transformation and type VI secretion in *V. cholerae* have been discovered, however more factors remain to be identified. Specifically, while TfoX and CytR co-regulate many competence promoters they have not been shown to directly bind to gene promoters and thus additional factors likely exist connecting each regulatory protein to specific genes. Similarly, while QstR overexpression can complement the absence of TfoX, CytR

and HapR for activating the T6SS, the mechanism by which QstR activates T6SS is still poorly understood.

- In this study, novel effector classes have been identified some of which belong to the lipase family while others have no putative function. Prior evidence suggests that these effectors likely encode toxic anti-bacterial activity. *In vivo* characterization of these effectors will provide greater insights into their activity and potential mechanisms of action.
- *In vivo* demonstration of horizontal transfer of functional T6SS genes confirms prior predictions that T6SS alleles are frequently exchanged this way. Preliminary findings show that HGT events leading to exchange of T6SS alleles leads to community diversification and a complex set of pairwise antagonistic interactions that affect the survival of parental as well hybrid isolates. Similarly modeling efforts not included in this thesis show that horizontal acquisition of T6SS alleles is beneficial from an evolutionary standpoint when *Vibrio cholerae* faces competition with superior T6SS weaponry. Future studies involving multi-partner competitions will help further characterize the impact of frequent HGT events that modify the T6SS repertoire of *Vibrio cholerae*.

The findings discussed in this dissertation have advanced the scientific understanding of processes contributing to chitin utilization, natural transformation and type six secretion in *V. cholerae* as well as shedding light on the complex interplay between these phenotypes. In addition this thesis explores the impact of these interactions on communities of different *V. cholerae* cells competing in the same spatial environment. Because transformation and type six secretion are important mechanisms for survival and

adaptation, these studies have begun to define a role for how and why *V. cholerae* utilizes these phenotypes in their natural environment and how it may contribute to the evolution of the species as a whole. Perhaps the work described here and future studies that arise from these findings will lead to a better understanding of how this human pathogen remains competitive in both the environment and human host.

APPENDIX A. Supplementary information for chapter two

A.1. Supplementary figures for Chapter 2

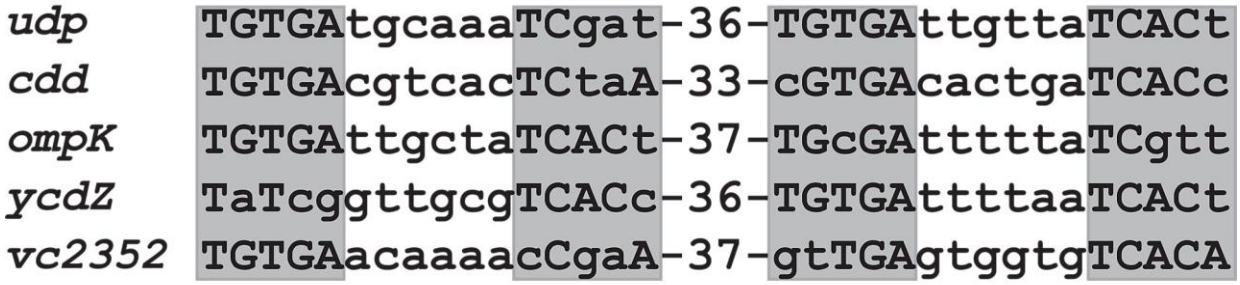


Figure 19: Predicted CRP binding site pairs of nucleoside catabolism and transport genes anti-activated by CytR.

Putative CytR binding sites were determined by identifying two CRP binding sites (highlighted in grey) in the -200 to +100 region (with respect to translational start site) of each target gene separated by a spacing of 49–53 nucleotides. CRP sites were determined by FIMO (C.E. Grant, T.L Bailey and W.S. Noble, Bioinformatics 27:1017–18, 2011) TGTGA-N6-TCACA ($p < 0.01$).

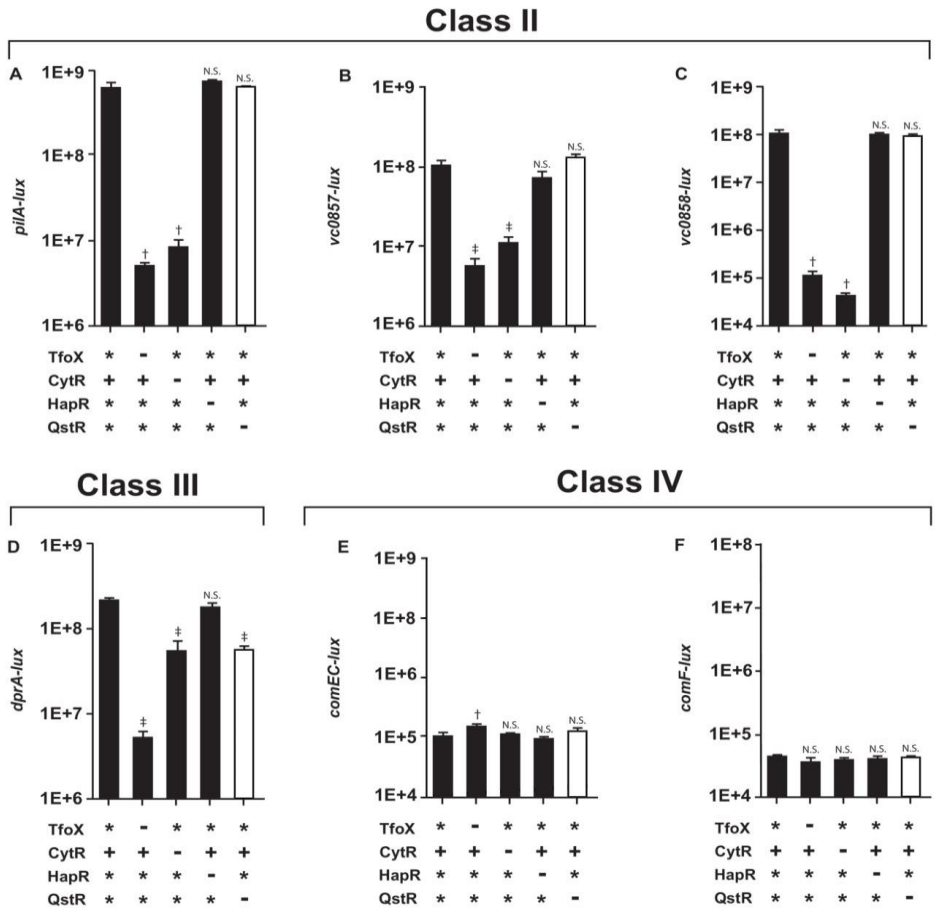


Figure 20: Differential regulation of competence genes by TfoX, CytR, HapR, and QstR.

V. cholerae strains in which the regulators *tfoX*, *qstR*, *cytR* and *hapR* were present (+), deleted (-) or constitutively induced (*) were analyzed for bioluminescence from the following plasmid-encoded transcriptional reporters: *pilA-lux* (Panel A), *vc0857-lux* (Panel B), *vc0858-lux* (Panel C), *dprA-lux* (Panel D), *comEC-lux* (Panel E), and *comF-lux* (Panel F). Bioluminescence is represented as relative light production per OD_{600} (RLU). Data shown are mean values ± standard deviation for biological triplicates. ‡ indicates a p-value < 0.01, † indicates a p-value <0.05. N.S. denotes not significant, calculated using a two-tailed Student's t-test. Bars 2–5 are compared to bar 1.

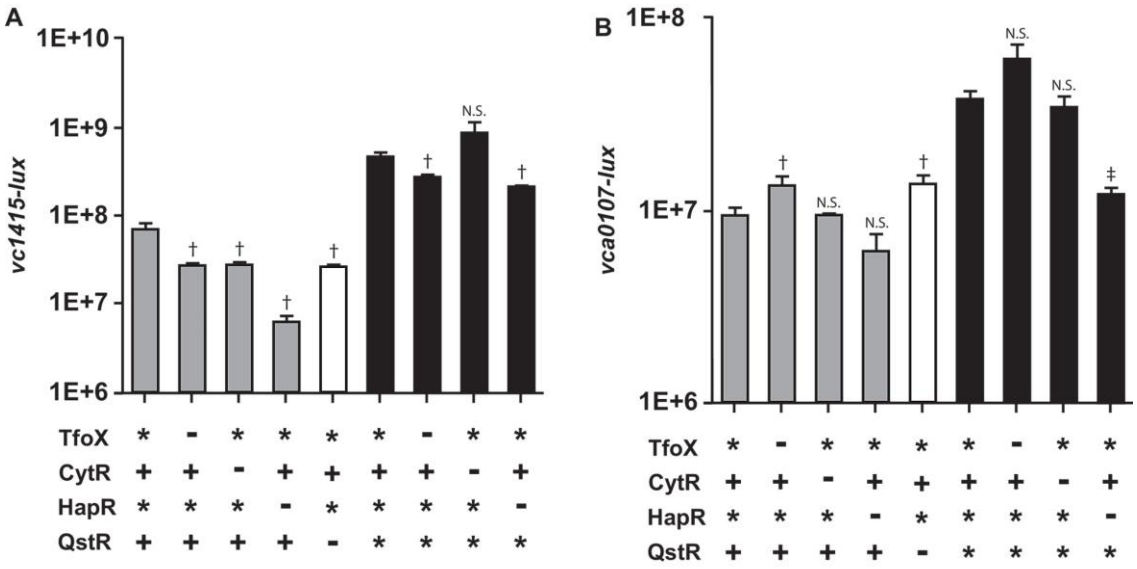
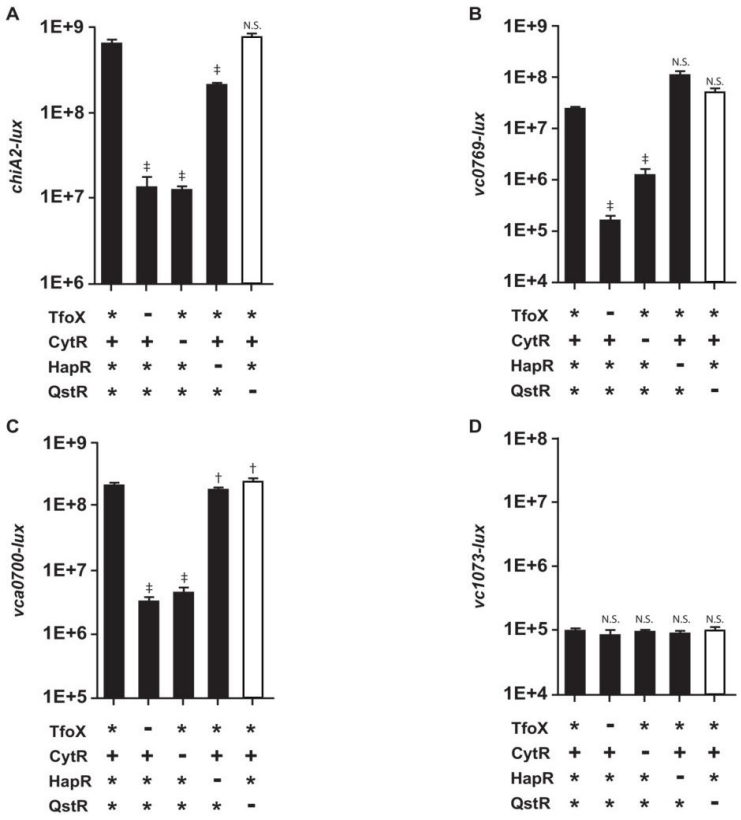


Figure 21: Differential regulation of T6SS clusters by TfoX, CytR, HapR, and QstR.

V. cholerae strains carrying the indicated alleles of the regulators tfoX, qstR, cytR and hapR (+, native;-, deletion; *, constitutively expressed) were analyzed for bioluminescence from the plasmid-encoded transcriptional reporters vc1415-lux (Panel A) and vca0107-lux (Panel B). Bioluminescence is represented as relative light production per OD600 (RLU). Data shown are mean values ± standard deviation for biological triplicates. ‡ indicates a p-value < 0.01, † indicates a p-value <0.05. N.S. denotes not significant, calculated using a two-tailed Student's t-test. Bars 2–5 are compared to bar 1 and bars 7–9 are compared to bar 6.



E

genotype	colony diameter (mm) ± s.d.	zone of clearing diameter (mm) ± s.d.
*tfoX**	11.00 ± 0.01	14.50 ± 0.51
tfoX-	11.67 ± 0.49	< d.l.
tfoX cytR-*	10.00 ± 0.01	< d.l.
tfoX chiA1-*	10.22 ± 0.46	12.22 ± 0.01
tfoX chiA2-*	10.00 ± 0.01	11.00 ± 0.01
tfoX vc0769-*	10.50 ± 0.51	13.39 ± 0.70
tfoX vca0700-*	10.00 ± 0.01	13.11 ± 0.32
tfoX chiA1-chiA2-*	10.00 ± 0.01	< d.l.
tfoX chiA1-chiA2- vc0769-vca0700-*	10.44 ± 0.53	< d.l.
tfoX cytR+ hapR* qstR**	10.00 ± 0.01	14.00 ± 0.01
tfoX- cytR+ hapR qstR**	12.00 ± 0.01	< d.l.
tfoX cytR* hapR* qstR**	10.00 ± 0.01	< d.l.
tfoX cytR+ hapR- qstR**	10.44 ± 0.53	12.67± 0.50
tfoX cytR+ hapR* qstR-*	10.11 ± 0.31	15.00± 0.01

Figure 22: Differential regulation of chitinases by TfoX, CytR, HapR, and QstR.

V. cholerae strains in which the regulators *tfoX*, *qstR*, *cytR* and *hapR* were present (+), deleted (-), or constitutively induced (*) were analyzed for bioluminescence from the following plasmid-encoded transcriptional fusions to chitinase promoters: *chiA2-lux* (Panel A), *vca0700-lux*(Panel B), *vc0769-lux* (Panel C) and *vc1073-lux* (Panel D). Bioluminescence is represented as relative light production per OD_{600} (RLU). Data shown are mean values ± standard deviation for biological triplicates. ‡ indicates a p-

value < 0.01, † indicates a p-value <0.05. N.S. denotes not significant, calculated using a two-tailed Student's t-test. Bars 2–5 are compared to bar 1. (Panel E) Chitin agar plate assay. Indicated strains were assayed for the ability to degrade colloidal chitin and produce a visible zone of clearing. Measurements show representative values for mean colony diameter and zone of clearing as well as standard deviation obtained from 9 biological replicates for each strain tested.

A.2. Supplementary Tables for Chapter 2

Table 4: Summary statistics of RNA-seq.

12 Multiplexed cDNA libraries were derived from DNA-depleted *Vibrio cholerae* total RNA and sequenced to give 100 bp paired end reads as described in Materials and Methods. >98% of the 216 million reads obtained mapped onto the reference genome of *Vibrio cholerae* N16961 (J. F. Heidelberg, J. A. Eisen, W. C. Nelson, R A. Clayton, et al. Nature 406(6795): 477–483.) obtained from EBI.

Sample Name	**Genotype**	**Index**	**Yield (GB)**	**# Reads (Millions)**	**% Reads Q >=30**	**Mean Q Score**	**% Reads aligned to reference genome**
A	Δ*luxO*, *tfoX**, Δ*cytR*, Δ*lacZ*:*hapR*	ATCACG	1.89	18.89	86.7	34.12	99.18
B	Δ*luxO*, *tfoX**, Δ*cytR*, Δ*lacZ*:*hapR*	CGATGT	2.11	21.09	87.36	34.3	98.79
C	Δ*luxO*, *tfoX**, Δ*cytR*, Δ*lacZ*:*hapR*	TTAGGC	1.92	19.20	86.6	34.08	98.39
D	Δ*luxO*, *tfoX**, Δ*lacZ*:*hapR*	TGACCA	2.18	21.79	86.77	34.12	98.72

Table 4 continued

E	Δ*luxO*, *tfoX**, Δ*lacZ*:*hapR*	ACAGTG	1.76	17.56	86.87	34.15	98.6
F	Δ*luxO*, *tfoX**, Δ*lacZ*:*hapR*	GCCAAT	1.9	18.98	86.91	34.17	98.44
G	Δ*luxO*, Δ*cytR*, Δ*lacZ*:*hapR*	CAGATC	1.8	18.02	87.43	34.31	99.41
S	Δ*luxO*, Δ*cytR*, Δ*lacZ*:*hapR*	ACTTGA	1.81	18.05	87.46	34.32	99.39
I	Δ*luxO*, Δ*cytR*, Δ*lacZ*:*hapR*	GATCAG	1.45	14.46	87.76	34.42	99.44
J	Δ*luxO*, Δ*lacZ*:*hapR*	TAGCTT	1.55	15.53	87.5	34.33	99.39
K	Δ*luxO*, Δ*lacZ*:*hapR*	GGCTAC	1.74	17.43	87.64	34.38	99.41
L	Δ*luxO*, Δ*lacZ*:*hapR*	CTTGTA	1.51	15.14	87.82	34.42	99.3
		Total	21.62	216.14			

Table 5: List of strains and plasmids used in this study.

Strains	**Genotype or Description**	**Reference**
SW051	*ΔluxO ptac-tfoX ΔcytR ΔlacZ:HapR*	This study
EA349	*ΔluxO ptac-tfoX ΔlacZ:HapR*	E.S. Antonova, E. E. Bernardy and B. K. Hammer, Mol. Microbiol. **86**:1215-31, 2012
EA432	*ΔluxO ΔcytR ΔlacZ:HapR*	This study
EA372	*ΔluxO ΔlacZ:HapR*	This study
EA281	Δ*luxO ptac-tfoX*	E.S. Antonova, E. E. Bernardy and B. K. Hammer, Mol. Microbiol. **86**:1215-31, 2012
JT100	Δ*luxO ptac-tfoX ptac-qstR*	This study
JT708	Δ*luxO* Δ*tfoX*	This study
JT710	Δ*luxO* Δ*tfoX ptac-qstR*	This study
EA637	Δ*luxO ptac-tfoX* Δ*cytR*	E.S. Antonova, E. E. Bernardy and B. K. Hammer, Mol. Microbiol. **86**:1215-31, 2012
JT98	Δ*luxO ptac-tfoX* Δ*cytR ptac-qstR*	This study
JT128	Δ*luxO* Δ*hapR ptac-tfoX*	This study
JT127	Δ*luxO* Δ*hapR ptac-tfoX ptac-qstR*	This study
JT129	Δ*luxO* Δ*qstR ptac-tfoX*	This study

Table 5 continued

EA90	*lacZ:KanR*	E.S. Antonova and B.K. Hammer, FEMS Microbiol. Lett. **322**:68-76, 2011
EA305	*ptac-tfoX*	This study
EA355	*ΔtfoX*	This study
EA431	*ptac-tfoX ΔcytR*	This study
SW323	*ptac-tfoX-Chr ΔchiA1*	This study
SW336	*ptac-tfoX-Chr ΔchiA2*	This study
SW352	*ptac-tfoX Δvc0769*	This study
JT603	*ptac-tfoX ΔvcA0700*	This study
SW358	*ptac-tfoX-Chr ΔchiA1 ΔchiA2*	This study
SW408	*ptac-tfoX-Chr ΔchiA1 ΔchiA2 Δvc0769*	This study
SW410	*ptac-tfoX-Chr ΔchiA1 ΔchiA2 Δvc0769 Δvca0070*	This study
NRD204	*E. coli* MG1655 Δ*araBAD::cat*	N. R. De Lay and J. E. Cronan, J. Biol. Chem. **282.28**: 20319-28, 2007
C6706str2	C6706 El Tor biotype O1	K. H. Thelin and R. K. Taylor, Infect. Immun. **64(7):** 2853-2856, 1996
JT652	Δ*luxO ptac-tfoX ptac-qstR ΔvasK*	This study

A.2.1. Supplementary dataset

Table 6: Differential regulation of genes by CytR and by TfoX in *V. cholerae*.

Raw counts of the transcripts obtained from coding regions were calculated and pairwise comparisons were made to calculate relative fold-change as described in the text. A fold change >2 and a p value <0.05 denotes positive regulation while a fold change <0.5 indicates negative regulation. Data in each tab of the spreadsheet refers to individual columns described in Figure 8 of the main text.

APPENDIX B. Supplementary information for chapter three

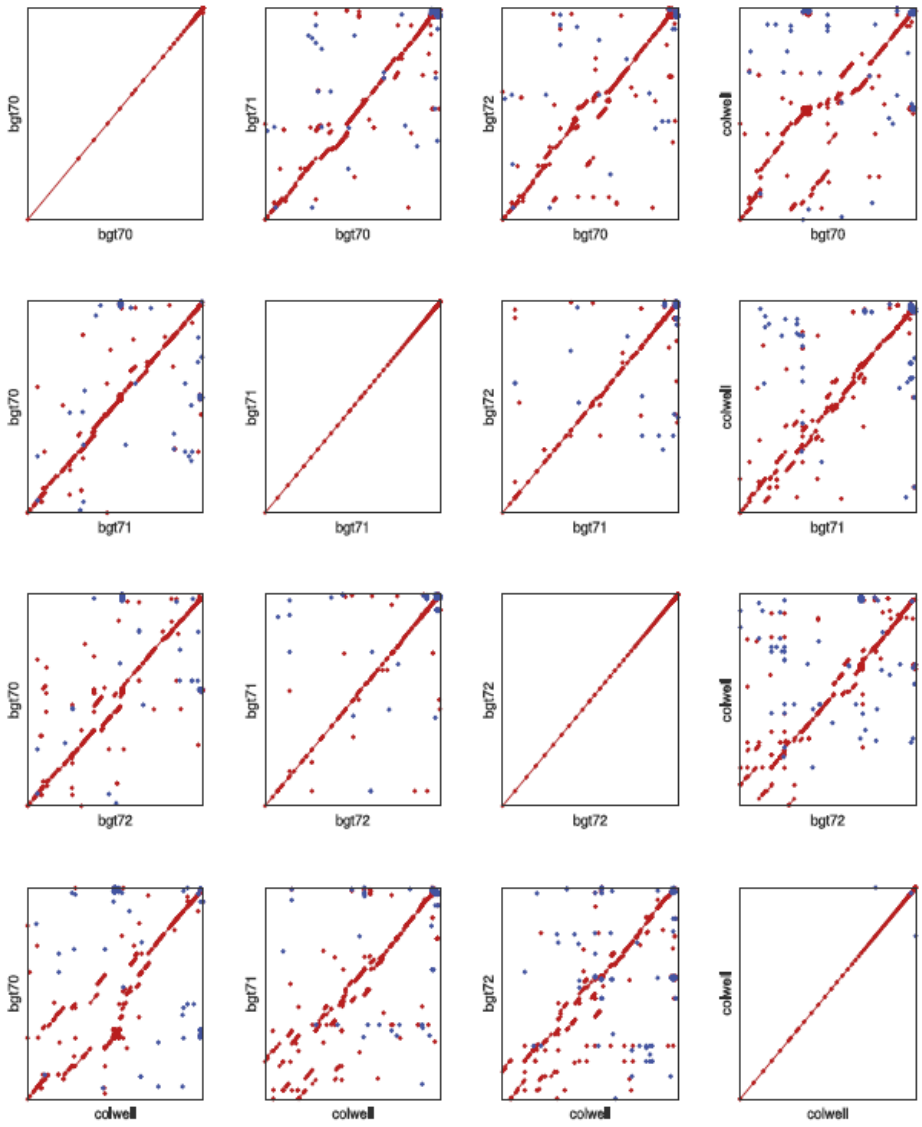


Figure 23. Dot plots of ANI group 4 genomes.

Pairwise dot plots of 4 genomes, BGT61, 70, 71 and EGT01. BGT61, 71 and 72 have several, small rearrangements and unique regions, consistent with high rates of HGT. EGT01, while close by ANI, contains many rearrangements and unique regions.

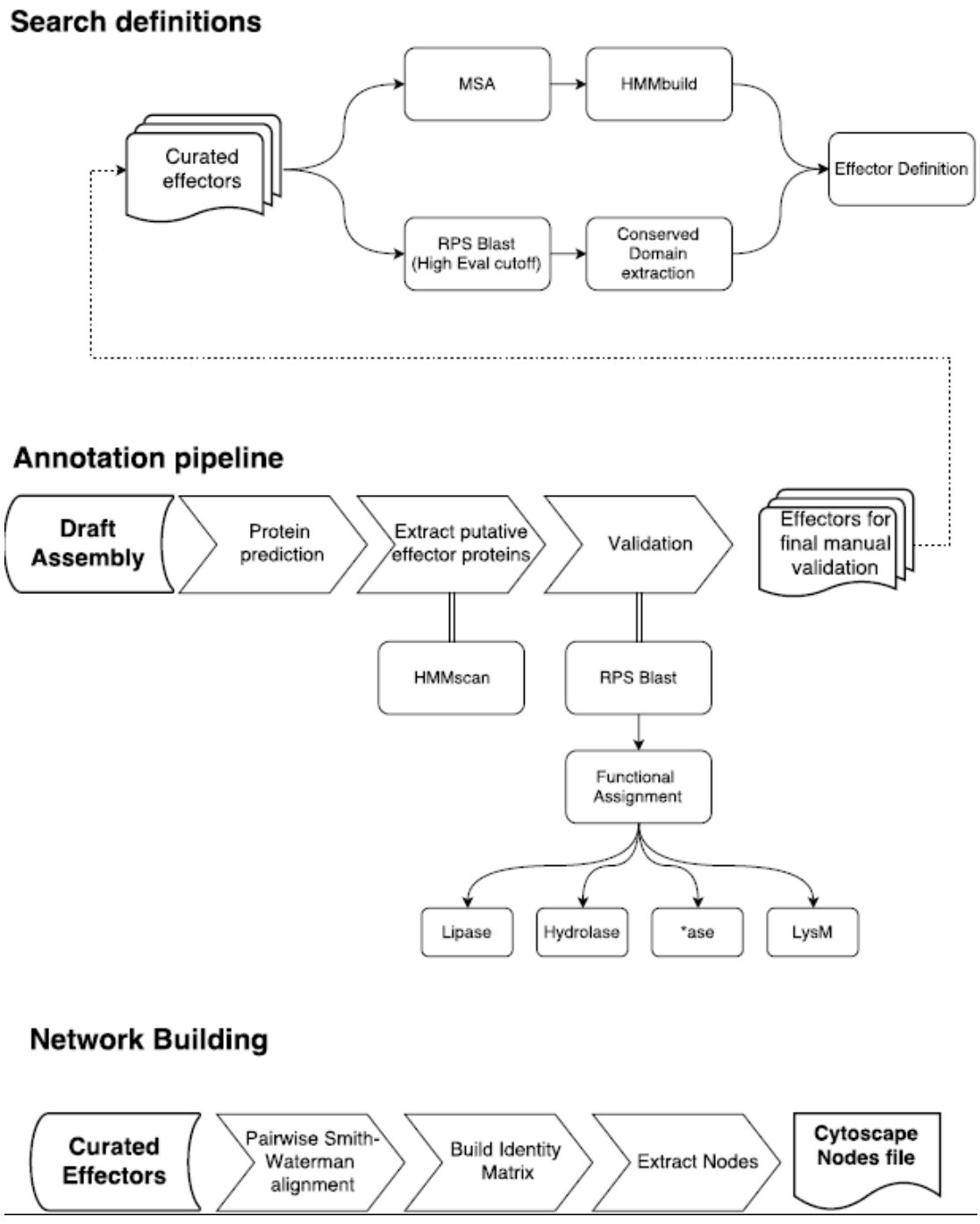


Figure 24. Annotation workflow.

Graphical description of the annotation workflow used in this study and, in part, by T6SS predictor.

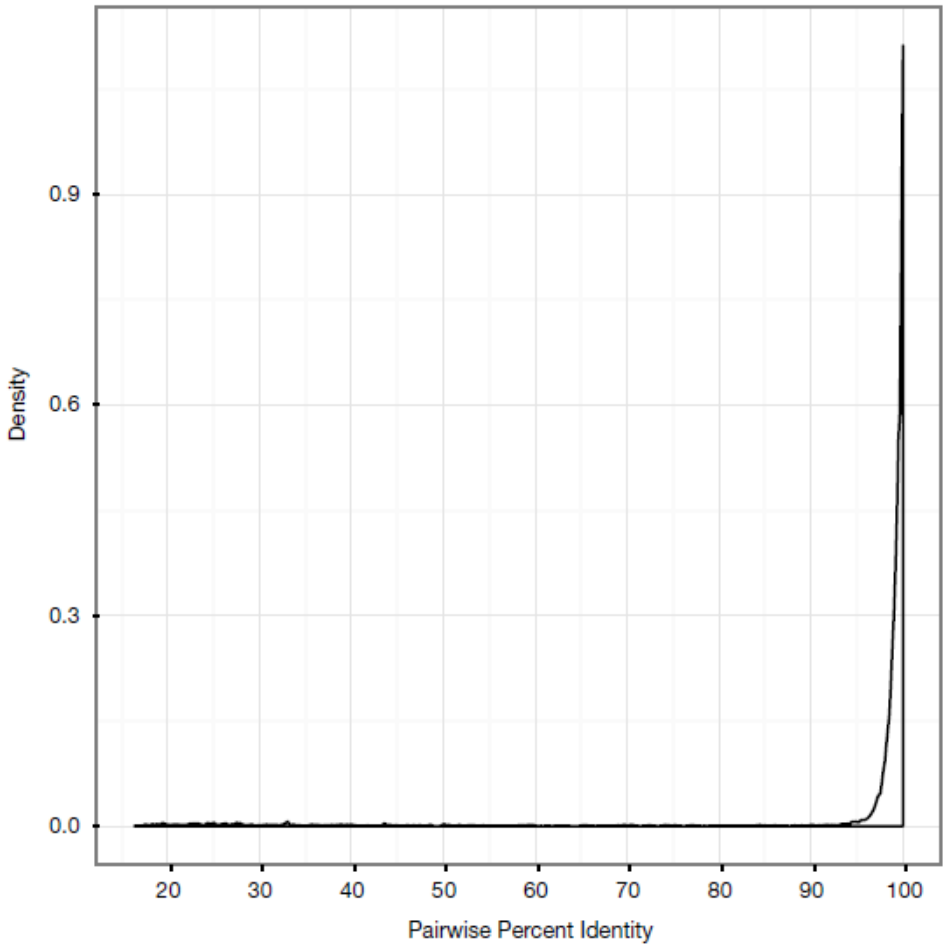


Figure 25. Distribution of sequence identity between same annotations.

Distribution of pairwise sequence identity between two genes with matching annotations between genomes. Approximate 276,000 comparisons are shown; >99.99% of density is represented in the 95-100% identity region.

APPENDIX C. Supplementary information for chapter four

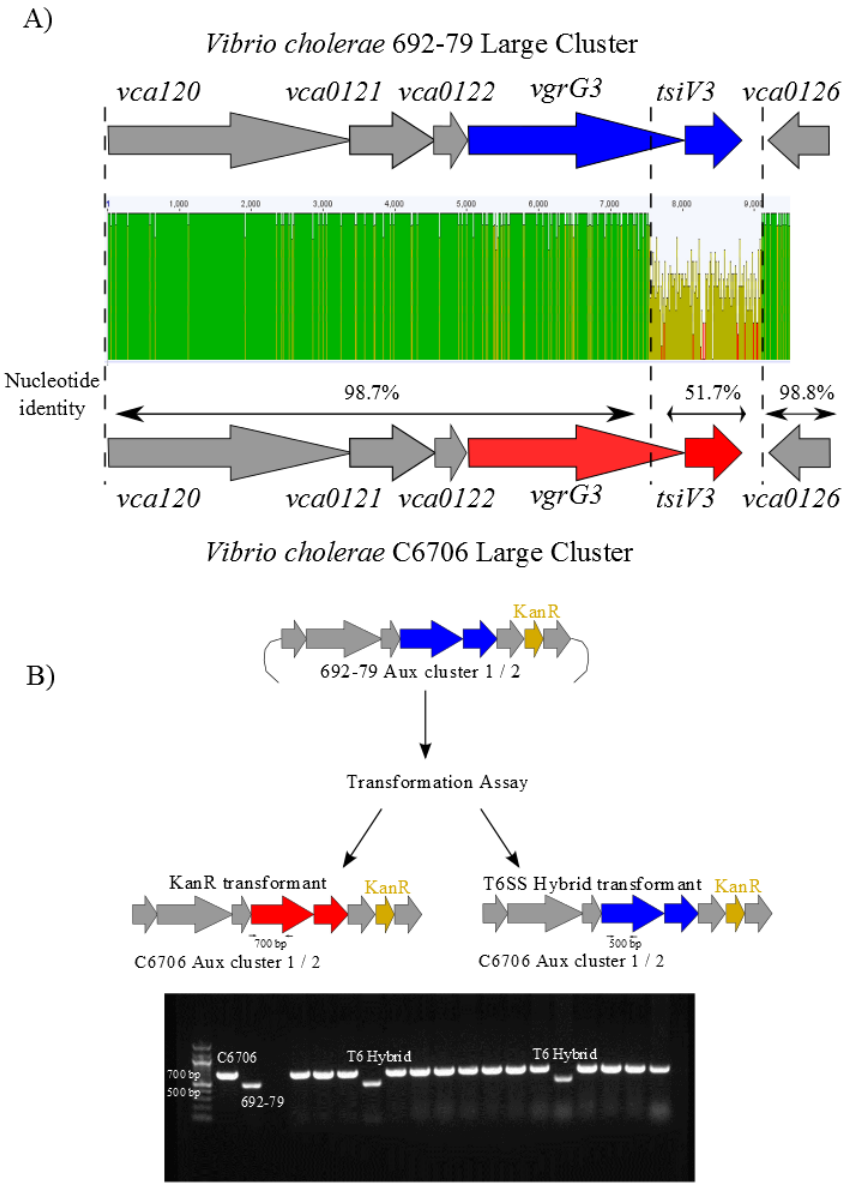


Figure 26. Nucleotide comparisons between T6SS large clusters of C6706 and 692-79 and Schematic of transformation assay.

A) Pairwise nucleotide alignments were performed between type six secretion system large cluster from an environmental (non-toxicogenic) isolate 692-79 and clinical (toxicogenic) isolate C6706. Both clusters show low sequence conservation in the effector/immunity loci (middle) flanked by high conservation upstream and downstream

indicating that the two sequences might have distinct origins. B) Schematic of the transformation assay. Downstream regions of T6SS Auxiliary cluster 1 and 2 of environmental isolate 692-79 were marked with a KanR cassette and gDNA isolated from these strains was used as eDNA for transformation experiments. Transformants that horizontally acquired T6SS genes from the environmental isolate (blue) along with the selectable marker were distinguished from those acquiring only the selectable marker by performing allele specific PCR using primers indicated above. A ~500 bp band indicates acquisition of T6SS genes by the recipient.

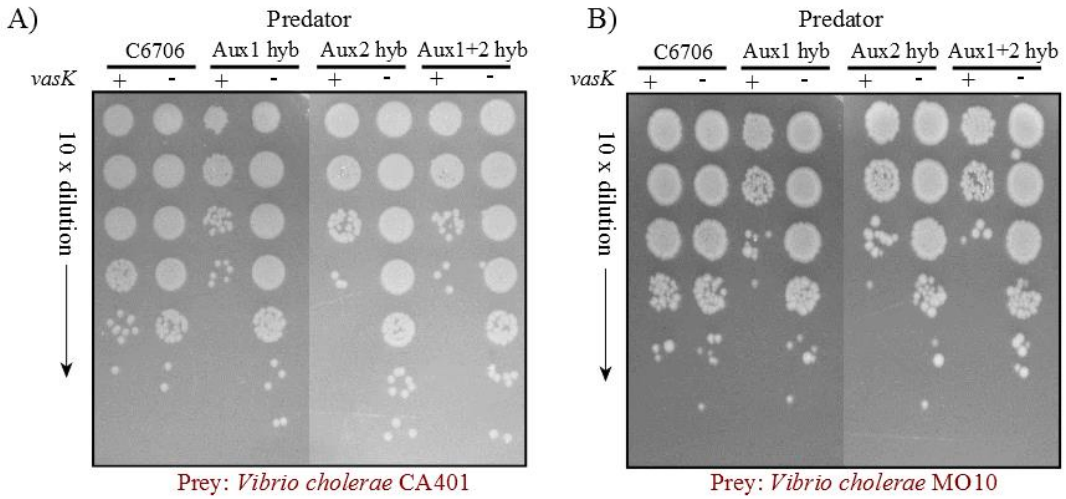


Figure 27. T6SS hybrids can effectively kill clinical isolates CA401 and MO10

A) *V. cholerae* CA401 and B) *V. cholerae* MO10 isolates shows decreased survival when competed against H1, H2 and H1+2 hybrid predators relative to C6706 predator.

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